An investigation into the composition and nutritional properties of a mixed seed food product

Khadija Samir Radhi

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School of Life Sciences

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Abstract

Maintaining an adequate food supply remains a global challenge, with hundreds of thousands of lives lost each year because of malnutrition, especially in developing countries. Although malnutrition tends to be more common in these countries, where there are shortages of food, in industrialised countries, more and more people are being diagnosed with malnutrition caused by food allergies. The main approach to managing allergies is to avoid certain foods, which may also lead to inadequate food intake and malnutrition. The first aim of this study was to develop a nutritional, ambient-stable, confectionary product containing seeds, nuts, and treacle with raw materials that are readily available in Middle Eastern and African countries. The second aim was to determine the effect of different thermal processing methods on the product with respect to Maillard reaction products (MRP) and antioxidant properties. The third aim was to measure the effect of different thermal processing methods and additives on the composition, solubility, structure, and immune reactivity of peanut allergens extracted from the product. The fourth aim was to test the effect of additives such as sodium bisulphite and ascorbic acid on the structure and allergenicity of peanut proteins. The second chapter of the thesis addresses product development based on literature-derived information of the proximate composition of various ingredients, including peanuts, sesame seeds, Nigella seeds, and treacle. The composition of the final product, called the black seed mix, was analysed by proximate analysis using approved AOAC methods. The amino acid composition was analysed by HPLC and the fatty acid composition by GC and minerals by atomic absorption spectroscopy. It was demonstrated that the product contains significant quantities of fat, carbohydrate, fibre, linoleic acid, protein and essential amino acids, magnesium, zinc and iron, to meet nutritional requirements for selected age groups. The third chapter deals with shelf stability and sensory analysis of the product. Water

activity measurements of the fresh product, combined with knowledge of sugar content, led to categorisation of the product as a chewy, sweet confection. Texture analysis of the fresh product fell within an acceptable range, and the sensory evaluation proved to be acceptable. The vacuum -packed product was stable after one month's storage under accelerated storage conditions (37°C). However, after 2 month's storage, the product showed increased water activity, texture hardness, fracturability and cohesion strength, but this was still within an acceptable range according to published criteria. The peroxide value of fats also increased after 2 months accelerated storage, which was regarded as a limiting factor. The fourth chapter addresses the effect of different processing conditions on extracts of the black seed mix on Maillard reaction products (MRP; measured by spectroscopic absorbance and fluorescence), antioxidant activity (radical scavenging, ferric reducing and inhibition of lipid peroxidation measured by DPPH, FRAP, FTC, and TBA methods), and solubility (Bradford and Kjehldahl methods). The roasted product showed increased third stage MRP, which correlated with improved radical scavenging activity, similar ferric reducing activity but reduced inhibition of lipid peroxidation ability compared to the control and boiled sample. The boiled product resulted in higher intermediate-stage MRP, increased radical scavenging ability, reduced ferric reducing ability and increased inhibition of lipid peroxidation ability. The fifth chapter deals with the effect of processing and the addition of additives to the black seed mix on immune reactivity of peanut allergens (peanut-allergic patient sera) using the ELISA, SDS-PAGE, and Western Blot techniques. The results indicate that extract from the roasted product caused increased immune reactivity. I also report the loss of soluble peanut 7S and 11S in extracts from the boiled version, leading to reduced immune reactivity for the soluble fraction (ELISA and Western blot). I demonstrated that the addition of the approved food additive sodium bisulphite to the black seed mix lowers the immune reactivity of peanut

allergens by ELISA, which was caused by reduction of disulphide bonds of the 11S peanut allergen as determined by SDS-PAGE. It is concluded that the preferred thermal processing method of the black seed mix would be boiling of the peanuts before addition to the rest of the ingredients and further processing.

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DEDICATED TO MY SON

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I offer my highest regards to the beautiful and majestic city of Edinburgh, this great city with noble people that I will never forget.

ACADEMIC REGISTRY

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Contents

List of Figures

List of Tables

Abbreviations

AAA	Aromatic amino acids
AGEs	Advanced glycation end products
BSA	Bovine serum albumin
BHA	Butylate hydroxyanalsoe
BHT	Butylated hydroxytoluene
^{o}C	Degrees Celsius
Ca	calcium
Cu	copper
CAT	Catalase
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EAACI	European Academy of Allergology and Clinical Immunology
FDA	Food and drug administration
Fe	Iron
FTC	Ferric thiocyanate
GPX	Glutathione peroxidase
HAT	Hydrogen donation
HBA	Thiobarabituric acid
His	Histidine
HIV	Human immunodeficiency virus
IDF	Insoluble dietary fibre
IgE	Immunoglobulin E
Ile	Isoleucine
Kcal	Calories
K	potassium
Leu	Leucine
Lys	Lysine
M	Molar
Mg	Magnesium
Mins	Minutes
MRPs	Maillard reaction products
MT	Metric ton
Na	sodium
PEM	Protein energy malnutrition

OPA	o-phthaldialehyde
P	Phosphorous
PBS-T	Phosphate buffered saline-tween
PBUH	The prophet Mohamed
Pb	lead
PG	Propylgallate
PUFA	poly-unsaturated faty acid
PV	Peroxide value
ROS	Reactive oxygen species
SAA	Sulphura mino acids
SB	Sodium bisulphate
SDF	Soluble dietary fibre
SDS	Sodium dodecyl sulphate
SDS-PAGE	Polyacrylamide Gel Electrophoresis
SOD	Superoxide dismutase
TBHQ	Teri- butylhydroquinon
TBP	Tributylphosphine
Thr	Hreonine
Trp	tryptophan
UN	United Nations News Centre
UNHCR	United Nations High Commissioner for Refugees
Val	valine
Wa	Water activity
WHO	World Health Organization

CHAPTER 1

General Introduction

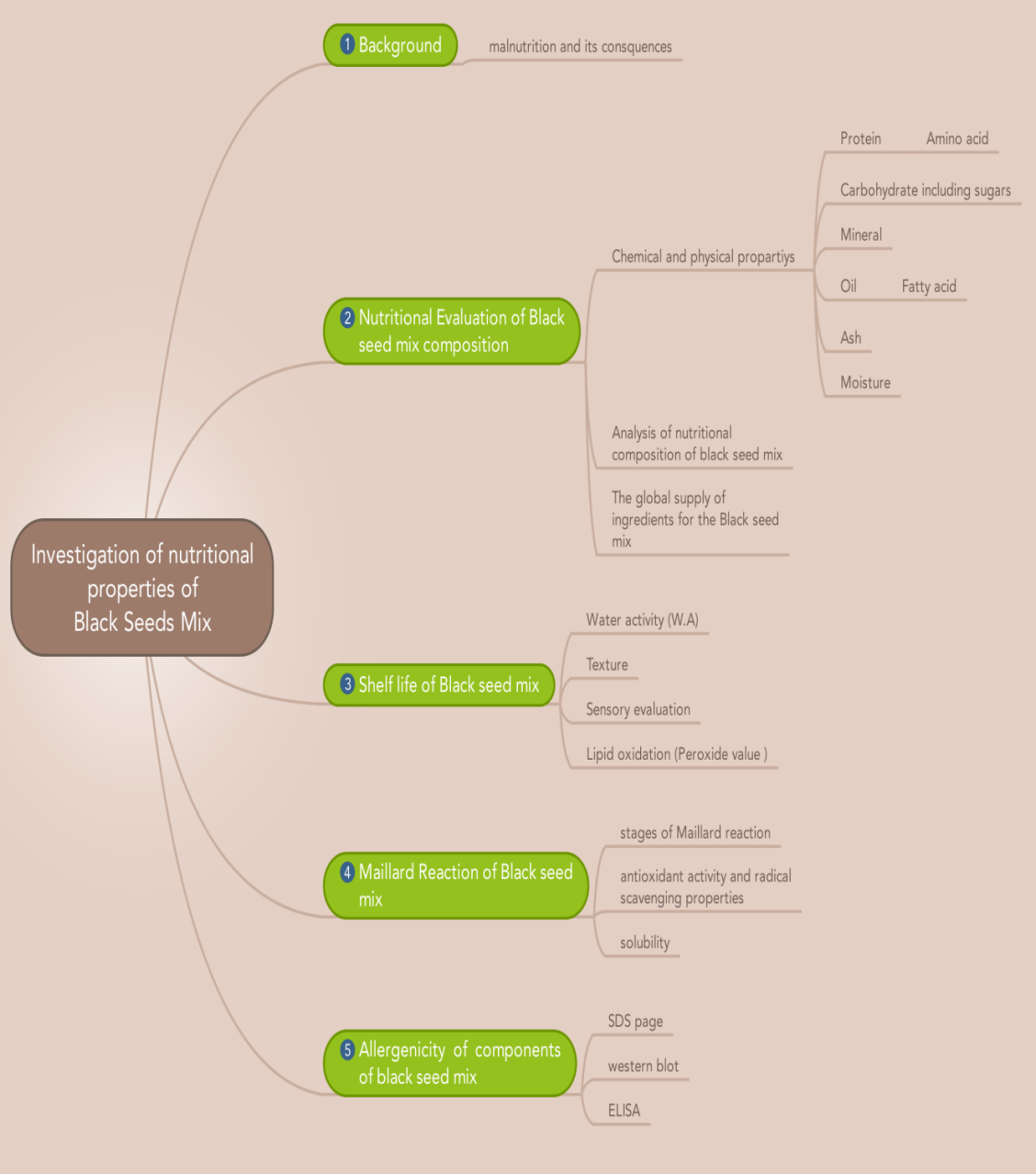


Figure 1.1. Lay out of the thesis

1.1 Aims of the project

The aims of this study were as follows:

1. Development of a nutritional, ambient-stable, confectionary product containing seeds, nuts, and treacle with raw materials that is readily available in Middle Eastern and African countries.

2. Determination of the effect of different thermal processing methods of the product on different stages of Maillard reaction products (MRPs) and antioxidant properties.
3. Measurement of the effect of different thermal processing methods on the composition, solubility, structure, and immune reactivity of peanut allergens extracted from the product.
4. Testing of the effect of additives such as sodium bisulphite and ascorbic acid on the structure and immune reactivity of peanut proteins.

1.2 Novelty of the research approach

1. An ambient-stable black seed mix consisting of raw materials in the traditional recipe that could serve as an adequate source of carbohydrate, fibre, protein, and essential amino acids for children and adolescents has not been reported in the literature. The traditional food product is widely made and consumed in households in Middle Eastern countries, and knowledge and optimisation of its composition could be useful as a source of nutrition for malnourished children.
2. Previous studies mainly report the effect of third-stage Mallard reaction products (MRPs) on radical scavenging activity and ferric reducing activity in model systems containing lysine and reducing sugars. The present study will investigate the effect of different thermal processing methods of a nut- and sugar-containing complex food matrix on (MRPs) with respect to radical scavenging, ferric reducing activity and inhibition of lipid peroxidation.
3. Previous studies on the immune reactivity of glycated peanut proteins have been carried out on isolated peanut proteins or peanut-sugar and peanut-polysaccharide mixtures. There are no reports on the immune reactivity of a mixture of proteins glycated in a complex waterless food matrix. The present study investigates the effect

of different thermal processing methods of the black seed mix on peanut immune reactivity.

4. A recent publication reported the reduction of immune- reactivity of allergens isolated from cashew nuts by addition of sulphite (Mattison, 2014). The present study investigates the effect the addition of sodium bisulphite or ascorbic acid to a complex food matrix on peanut protein immune reactivity and structure, which has not been reported.

1.3 Background to malnutrition

1.3.1 Global status of malnutrition

It is agreed that malnutrition is a global crucial matter. Maintaining an adequate food supply remains a global challenge, with hundreds of thousands of lives lost each year because of malnutrition, especially in developing countries. Based on reports of the World Health Organization, there are nearly two billion people all around the world that suffer from malnutrition (Science, 2014).

Africa bears a particularly severe burden, especially in Somalia, with drought, hunger, and malnutrition causing human devastation, not to mention severe economic losses (Gettleman, 2012). The UN has reported that over 29,000 children under the age of five died in 2012.

Malnutrition is also prevalent in large numbers of displaced people and refugees. The UNHCR's 2011 Global Report (UNHCR, 2011) reveals that many of the world's poorest countries are hosting huge refugee populations, both in general terms and in relation to the size of their economies. According to a report by the World Health Organization, there are about 21.5 million refugees as well as displaced people in different regions of the world, who are facing the problem of malnutrition (WHO, 2009). UNICEF recently led an inter-agency nutrition assessment on Syrian refugees in Lebanon and

reported that almost 2,000 Syrian refugee children in Lebanon are suffering from severe acute malnutrition, and need immediate treatment to survive. Another 8,000 Syrian refugee children in Lebanon are suffering from less severe forms of malnutrition. Syrian refugees are also at risk for anaemia, a condition where the oxygen carrying capacity of red-blood cells, or number of red blood cells, are inadequate; one of the major causes being lack of iron in the diet. To a lesser degree, anaemia also impacts refugees in Lebanon; 21 percent of Syrian refugee children under the age of 5 and 26.1 percent of Syrian refugee women of child-bearing age are inflicted with anaemia (Unicef, 2014).

Figure 1.2. Malnutrition in children under five years of age (Schaible and Kaufmann, 2007)

1.3.2 Causes and symptoms of malnutrition

The problem of malnutrition occurs when there is an inadequate utilization of energy and nutrients to maintain healthy and productive bodies. Malnutrition is characterized by both micronutrient deficiency and general malnutrition which is noticeable by stunted growth and low weight (WHO, 1999). Although symptoms of micronutrient deficiency and

malnutrition are manifested in a very complex way and also differ from one country to another, children are the most vulnerable part of the population (Rao *et al.,* 2005). Protein energy malnutrition (PEM) is responsible for most cases of death among children under five years of age (Rao *et al.,* 2005). Malnutrition affects the child's height, weight, and growth in general (Figure 1.2).

The case of pregnant women is seen as another group of concerns, considering the fact that mothers are at high risk of delivering babies that are prone to growth failure during their infancy due to the rate of breastfeeding. (de Onis *et al*., 2004). There is the risk that girls who are malnourished are at the risk of becoming malnourished themselves as mothers. Poor care for infants after birth in developing countries is reported to the cause of 23% of all deaths that occur among children who are less than five years old (de Onis *et al.,* 2004).

Many factors can contribute to high rates of child malnutrition, ranging from political instability and slow economic growth to the frequency of infectious diseases and the lack of education. Important determinants of child malnutrition, such as the prevalence of intrauterine growth retardation (IUGR) differ amongst countries and whether or not children are undernourished are determined by national factors and individual and household circumstances (de Onis, Blossner and Villar, 1998).

The main symptom of malnutrition is emaciation which occurs when a body lacks a large amount of necessary fat and particularly muscle tissue: it is a condition caused by lack of nutrients, starvation and/or disease (Stice *et al.,* 2004).

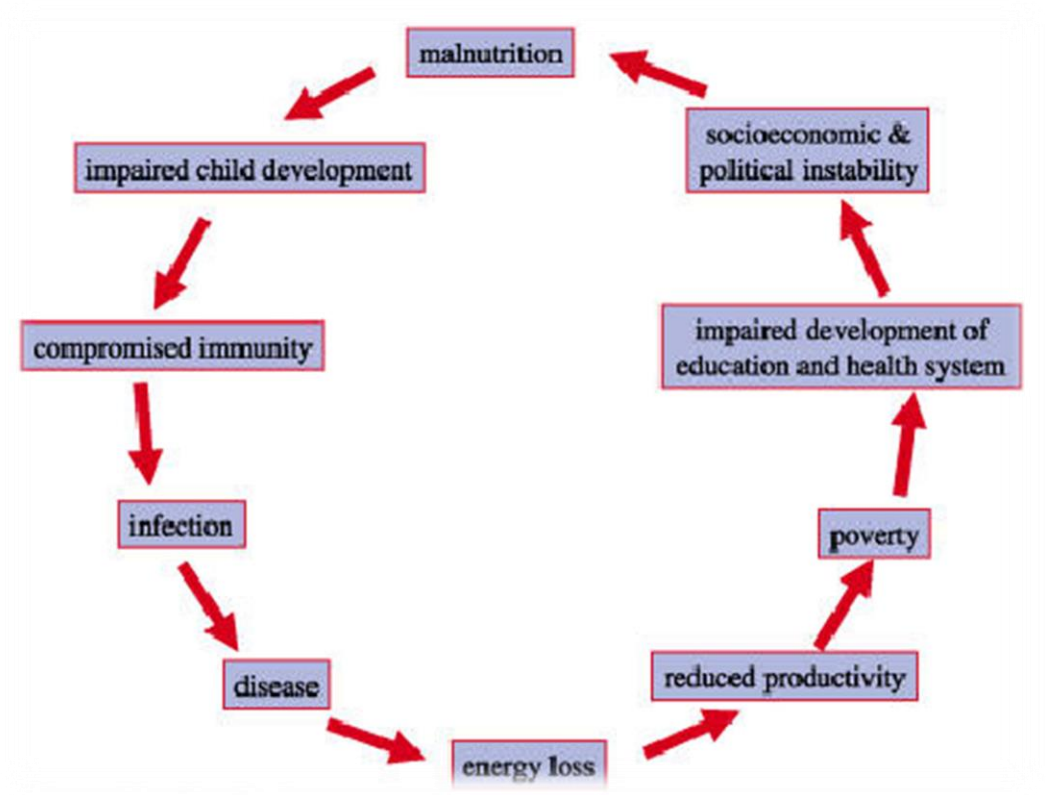


Figure 1.3. The consequences of malnutrition (HWO, 2014)

Emaciation is often defined as a body weight that is 25% less than the expected normal (Goette, 2005). Inadequate calories in the body can lead life-threatening conditions or diseases. Underweight conditions can also lead to changes in mood, weakening of the immune system and pervasive feeling of low energy (Goette, 2005). The most drastic malnutrition caused by protein-energy malnutrition (PEM) leads to either kwashiorkor or marasmus. The body may lose up to 60% of its normal weight and has a recognisable appearance, with an abdomen that is distended while the upper ribs are prominent. Vulnerability to disease is high with this illness, which is typically caused not only by inadequate food but also by the inadequate intake of high-protein foods (Goette, 2005) (Figure 1.3).

1.3.3 Malnutrition in developing countries

Although malnutrition tends to be more common in developing countries where there are shortages of food, in industrialised countries, more and more people are being diagnosed with malnutrition caused by diet, alcoholism, food allergies and eating disorders such as anorexia nervosa and bulimia nervosa. In the most severe cases, sufferers of these conditions become emaciated, with weights that are considered well below normal. Cancer and HIV can also result in striking weight loss and wasting of the muscles in a condition called cachexia (Goette, 2005). Malnutrition is also present in health care situations and even in well-developed health care systems, up to 50% of patients are malnourished (WHO, 2013). Shortages of iron, vitamin A, and zinc rank between the top ten primary reasons of death in developing countries (WHO, 2013).

1.3.4 Food allergies

The prevalence of food allergies is increasing while being better recognised (Mehta *et al.*, 2013). The avoidance of certain kinds of food is the major approach used in the management of allergies which leads to insufficient food intake and malnutrition (Meyer *et al*., 2013). People tend to avoid food that contain allergens such as milk and eggs, wheat, soy, peanut, tree nuts, fish, and shellfish. Growth monitoring and informing food allergic patients on suitable foods in order to supply essential nutrients are critical in avoiding deficiencies and growth retardation (Mehta *et al*, 2013). Most allergies in children are developed during their first 2 years of life. This is the critical period in the development and growth of children. Reports of poor growth and insufficient intake of nutrients in children having food allergy has been made, especially for children who avoid taking milk (Mehta *et al*, 2013).

1.4 Efforts to address malnutrition

The serious challenges facing the entire world as a result of malnutrition has led to many measures being put in place by the United Nations (UN), governments, many non-profit and non-government organisations through projects and special organizations to tackle the problem head on. A special session of the UN General Assembly on Children held in 2002 resulted in the formation of the Global Alliance for Improved Nutrition (GAIN). The aim of GAIN is to encourage public-private coalitions in handling issues of human sufferings brought about by malnutrition, and with specific focus on children, women, and girls. GAIN, through the joint undertakings of governments and international agencies are currently handling projects in 30 countries, and these are having positive effects on an estimated 667 million people. The objective of GAIN is to see to it that enough nutritious food reach a billion people (Global Alliance for Improved Nutrition, 2015). In 2009, the establishment of the Amsterdam Initiative against Malnutrition (AIM) took place during the GAIN Business Alliance Global Forum. This came about as a result of joint efforts of the Netherlands Ministry of Foreign Affairs and non-profit organisations (GAIN), together with prominent businesses such as Unilever and similar global companies. The goal is to eradicate malnutrition by 2015 among 100 million people in the continent of Africa (Global Alliance for Improved Nutrition, 2013). In 2000 the world's largest private foundation was established, Bill and Melinda Gates Foundation (BMGF), which spent an approximate amount of five million dollars for its 2014 programmes (Bill and Melinda, 2015). One of the world's oldest non-government organisations is the Save the Children which was established in 1919 in the United Kingdom. Save the Children currently has representations in over 120 countries and is responsible for providing direct help to 17.4 million children worldwide in 2014 (Save the Children UK, 2014).

In 1968, an Irish organisation known as Concern Worldwide (Concern), which has its focus directed in challenging hunger among the poorest people of the world, was established. The work of the organisation is being undertaken in approximately 25 countries located in regions of Africa, Asia, and the Caribbean (Concern Worldwide, 2013).

In 2010, an organisation known as the Thousand Days was established. The main aim of this organisation is to attain progress in the area of nutrition and to see to it that women and children are guaranteed that their first 1,000 days which come between pregnancy and the second birthday of the baby are the healthiest. This programme is a joint effort of the governments of the United State and Ireland, as well as GAIN, the Bill & Melinda Gates Foundation, and many other non-profit organizations (1,000 Days, 2010).

In 1977 the United Nations Standing Committee on Nutrition (UNSCN) was created, which is playing the role in encouraging cooperation among the agencies of the United Nations and partner organisations in supporting community and national, as well as regional and international endeavours to put an end to all forms of malnutrition hounding this generation (Unscn.org, 2009).

All these global efforts have made it possible for malnutrition in children to decline throughout the 1990s, resulting in the underweight prevalence in children to decline from 27% to 22% (de Onis *et al*., 2004). Eastern Asia recorded the largest reduction in the level of child malnutrition as underweight levels declined between the period 1990 and 2000 by 50%. Underweight rates in children living in south-eastern Asia declined from 35% to 27% while in Latin America and the Caribbean the incidence of underweight children declined at least one third over the last decade from 9% to 6%. However, Africa witnessed an increase in the number of underweight children from 26 million to 32 million during

the period between 1990 and 2000. The record also showed that 25% of all children below the age of five years old are underweight demonstrating that not much has changed from the previous 10 years (de Onis *et al*., 2004).

CHAPTER 2

Material and Methods

2.1 Materials

Ingredients

The ingredients of black seed mix (treacle, black seed, sesame, millet, peanut and fenugreek) were obtained from Saudi Arabia.

Chemicals

The following chemicals ingredients were obtained from Sigma- Aldrich: hexane, hydrochloride, amyloglucosidase, pancreatic solution; α-amylose hexane, acetic acid o; chloroform, potassium iodide, Iodine monochloride reagent , starch indicator, and sodium thiosulphate, albumin, Bradford solution, *o*-phthaldialdehyde regain, 2-mercaptoethanol, ethanol, linolenic acid, ammonium thiocyanate, 2-thiobarbituric acid and trichloroactic acid.

Kit for protein extraction

ReadyPrep™ Protein Extraction Kit (Total Protein) from Bio-rad (cat. no.: 163-2086)

Reagents for Enzyme-linked immunosorbent assay (ELISA)

Phosphate-buffered saline (PBS) from Sigma (cat. no.: P4417)

Peanut allergy human serum from Seralab (cat. no.: M4092313)

Anti-human IgG (whole molecule) – produced in goats conjugated to alkaline phosphatase was obtained from Sigma (cat. no.: A9919)

p-Nitrophenyl Phosphate Liquid Substrate System from Sigma (cat. no.: N 7653)

Reagents for Western blot

Novex® Sharp Unstained Protein Standard from Life Technologies (cat. no.: LC5801).

NuPAGE® Novex® 12% Bis-Tris Protein Gels from Life Technologies (cat. no.: NP0341PK2)

Transfer Stack, PVDF, and Mini from Life Technologies (cat. no.: IB4010-02)

SIGMA*FAST*ä BCIP/NBT tablet from Sigma (cat. no.: B5655)

2.2 Methods

2.2.1 Development of recipe and method of preparation of the black seed mix

The chemical composition of all ingredients was researched in the literature. A table was constructed to combine compositions of components and to calculate the proximate composition of the end product. From the results a recipe was developed for the Black seed mix product studied in this thesis.

The method used to prepare the black seed mix is shown in the diagram below (Figure 2.1)

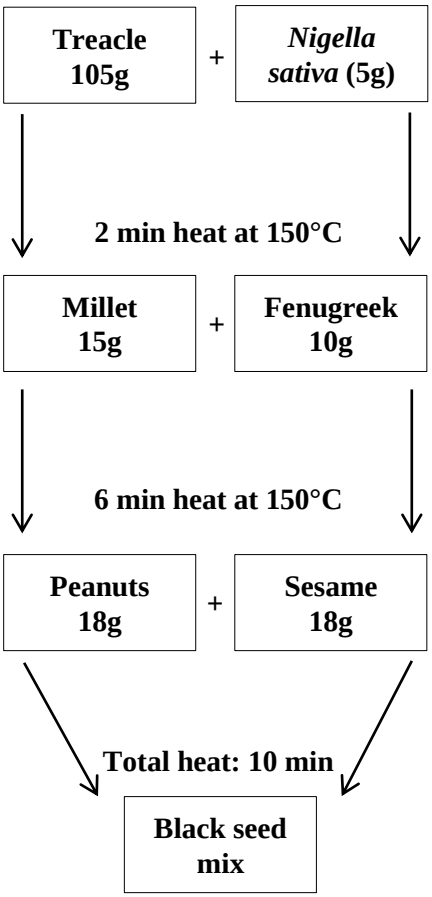


Figure 2.1. Preparation of the black seed mix.

The ingredients for the black seed mix were obtained from a mill in Jeddah, Saudi Arabia, while the treacle was obtained from a local shop in Jeddah. The ingredients were divided into three groups: 1) 105g treacle (sugarcane honey) that was mixed with 5g

Nigella sativa ;2) 10g fenugreek and 15g millet that was ground together; 3)18g peanuts that were crushed using a mortar and pestle and mixed with 18g raw sesame seed. The mixtures were stored in sealed containers at room temperature (between 18°C and 27°C).

The treacle mix (1) was poured into a separate pot and heated to 150°C. The millet and fenugreek (2) were added to the treacle after 2 min of heating and stirred until it dissolved completely and the mixture was thick. This mixture was stirred continuously and reheated until it reached 150°C. After 6 min, the crushed peanuts and sesame (3) were added and mixed until the mixture was homogeneous. The total heating time for the mix was 10 minutes (Figure 2.1). Finally, the mixture was left to cool to room temperature before being poured into a glass dish or pot and cooled. The finished product was stored in a closed container at room temperature.

2.2.2 Determination of the composition of the black seed mix

2.2.2.1 Moisture content

The moisture content of the black seed mix was determined by drying 1 g in an oven at 105°C overnight and then put in a desiccator to cool to room temperature. All measurements were replicated three times, the procedure given in (AOAC, 1990).

2.2.2.2 Ash

To determine the ash content, a sample of 3 g was dried in an oven overnight. Then the small sample was placed in a muffle at 500-550°C overnight to produce ash (Larrauri *et al.,* 1996). All measurements were replicated three times, and the ash percentage was calculated with the following equation:

Ash % = Weight of ash / Weight of sample ×100.

2.2.2.3 Oil

To determine the oil content, a 3 g sample was dissolved with 30 ml of water and freeze-dried. The powder sample was used to determine the oil content (AOAC, 1995). A 15 g sample was added to the extraction tube of a Soxhlet apparatus. About 300 ml of hexane was added to the 500 ml bottom flask of the apparatus, which was connected to the Soxhlet apparatus at 60ºC. The fat was extracted by running hexane over the sample at a rate of 3-4 drops per sec for about 10 h. A rotary evaporator was used for 10 min to remove the hexane that was mixed with the oil. All measurements were replicated three times, and the fat percentage was calculated according to the following equation:

Fat % = Weight of fat in sample / Weight of sample × 100.

Figure 2.2. Sample for oil extraction

Figure 2.3. Oil extracted from sample

2.2.2.4 Carbohydrate (including sugars)

The carbohydrate content was estimated by calculating (total solids – (protein + lipids + minerals), as described by Salma *et al.* (2007).

2.2.2.5 Minerals

For the determination of minerals, 3.83g of ash was digested with 5 ml of HCl and placed in a volumetric flask. 100 ml of distilled water was added, Measurements were made in triplicate and the mineral content was determined by atomic absorption (AOAC, 2000).

2.2.2.6 Soluble and insoluble dietary fibre

The SDF and IDF were determined by the enzymatic gravimetric AOAC method (Goni *et al.*, 2009).

2.2.2.7 Total insoluble dietary fibre (IDF)

The total IDF was determined by adding a sample of 0.1 g to 50 ml centrifuge tubes and then adding 10 ml of 0.1 M phosphate buffer (pH 7.5) to the sample. The pH was checked and adjusted to 1.5 with 1 M HCl Pepsin solution (0.2 ml of pepsin) was added to the sample, and then the sample was incubated at 40ºC for 1 hour. The pH was brought to 7.5 by adding 10ml of 1 M NaOH solution. 1 ml of pancreatic solution was added to the sample and then incubated at 37ºC for 6 hours. 10 ml of the phosphate buffer was added to the sample, and the pH was adjusted to 6.9 with 0.1M NaOH; then 1 ml of α-amylose solution was added. The sample was incubated at 37ºC for 16 hours and centrifuged for 15 min in 3,000 g. Then the supernatant was removed to use in the SDF method. The pellet was washed with 5 ml of distilled water, dried overnight at 105ºC, and then cooled in the desiccator and weighed. The value of the sample's weight is the insoluble dietary fibre (IDF). The measurements were made with duplicate samples (Goni *et al.*, 2009).

2.2.2.8 Total soluble dietary fibre (SDF)

SDF was determined by adding 10 ml of 0.2 M sodium acetate buffer pH 4.7 to 20 ml of the supernatant of the sample prepared above, followed by 0.1 ml of amyloglucosidase. The sample was incubated in a water bath constant shaker for 45 min at 60ºC. The solution was transferred to dialysis tubing (12,000-14,000 kDa molecular weight cut-off) and dialysed against water for 48 hours at 20ºC. The solution was removed from the dialysis tube and freeze-dried overnight. The powder samples are the values of the SDF in the sample. The measurements were made with duplicate samples (Goni *et al.*, 2009).

2.2.2.9 Protein

The protein content was determined by the Kjeldahl method, using a nitrogen conversion factor of 6.25 to crude protein in the following equation (AOAC, 2000). The measurements were made with duplicate samples:

Protein % = (Nitrogen) N x 6.38/sample * 100.

2.2.2.10 Amino acids

A Black seed mix sample of 10 mg was placed in a glass hydrolysis tube in an ice bath. 200 µl of cold performic acid was added and mixed in an ultrasonic bath for 10 min. The sample was stored overnight at -5°C. Then 0.8 ml of 7.5N HCl was added and mixed in a sonic bath for 15 min. The mixture was heated in a heating block in a fume cabinet at 110°C for 23 hours. After hydrolysis, the sample was cooled to room temperature. The tube was opened carefully and transferred to 5 ml volumetric flask and made up to 5 ml with water. Finally, the sample was filtered and dried in a rotary evaporator at 40°C, and the residue was dissolved in 800 µl of 0.2M sodium carbonate buffer, pH 9.7. Jim McKinley, of the School of Life Sciences (SLS), performed the amino acid analysis using high-performance liquid chromatography (HPLC). The measurements were made with 4 time samples (Mackey and Beck, 1982). Because acid hydrolysis destroys methionine, cysteine and tryptophan, the protein sample was treated prior to acid hydrolysis to convert methionine to methionine sulfone, and cysteine to cysteine. However, tryptophan requires a separated derivatisation method which was not applied, therefore tryptophan was not detected.

2.2.2.11 Fatty acids

Oil was extracted from the sample over an eight-hour period in a Soxhlet apparatus, using hexane as the solvent (2.2.2.3). Samples were analysed by gas chromatography using a

flame ionisation detector (FID; Isbell *et al*., 2008) by Jim McKinley, of the School of Life Sciences (SLS), the measurements were made in duplicate.

2.2.3 Shelf life tests

2.2.3.1 Preparation of samples

For the sensory evaluation, texture analysis, water activity and peroxide value tests, samples were prepared as described in Section 2.2.1. The cooled samples were sealed in plastic bags using a vacuum heat sealer and stored at 37^{o}C for two months. Each measurement was repeated 3 times and conducted on two samples for each time point of shelf life. The time points of shelf life were: a) fresh samples b) after one month and c) after two months storage.

Figure 2.4. The black seed mix after packaging

2.2.3.2 Determination of lipid oxidation and the peroxide value (PV)

2.2.3.2.1 *Fat extraction*

The black seed mix sample, at each point of storage, was defatted by using hexane as follows: Add 10 ml of water and dissolve it with 3 g of the sample. Add 10 ml of hexane

to the sample, stir the sample for 30 min at room temperature, and centrifuge at 500 g for 10 min (Wang *et al.,* 1999).

2.2.3.2.2 *Determination of the peroxide value (PV)*

The peroxide value (PV) was measured as follows: Add 3 g of the oil sample to a 250 ml Erlenmeyer flask, then add 10 ml of chloroform, and mix to dissolve the oil. Add 15 ml acetic acid, 1 ml of 15% potassium iodide (KI), and 200 µl of iodine monochloride (ICl) reagent, then mix and leave in the dark for 5 minutes. To the flask, add 30 ml distilled water and 1 ml starch indicator; a blue colour is observed. The solution was then titrated with 0.01M of sodium thiosulphate ($Na_2S_2O_3$) solution until the blue colour disappears. Set up the blank in another Erlenmeyer flask by adding 10 ml of chloroform, 15 ml of acetic acid, 30 ml of H_2O, and 1 ml of starch, and then titrate with $Na_2S_2O_3$ solution (Chindo *et al.,* 2010), All measurements were replicated three times.

Calculation of the peroxide value followed this equation:

PV= (V1-V0) ×T× 1.000/m (milliequivalents of peroxide available per 1 g of the sample)

V1 = sodium thiosulphate solution in the sample (ml)

V0 = sodium thiosulphate solution in the blank (ml)

T = titre of the thiosulphate solution (normality)

m = weight of the sample (g)

2.2.3.3 Texture measurements

The texture parameters of the product were determined by using a texture test for foodstuffs with the parameters of a Zwick/Roell-type Z010 machine texture analyser (BDO-FBO.TS, Ulm Germany), with a 9.9 mm diameter cylindrical probe, all measurements were replicated three times. The samples were analysed for hardness, fracturability and cohesion strength.

2.2.3.4 Sensory evaluation

A sensory evaluation of the fresh black seed mix sample was carried out according to Lustre *et al.* (2007). The analysis was performed by 11 panellists who were randomly selected from non-smoking the staff and students at the School of Life Sciences at Heriot Watt University in the UK. They were given training on the product and the conduct of the sensory evaluation. The evaluation of the black seed mix was based on an eight-point hedonic scale, with 1=disliked extremely, 2=disliked very much, 3=disliked moderately, 4=disliked slightly, 5=neither liked nor disliked, 6=liked slightly, 7=liked moderately, and 8=liked very much.

2.2.3.5 Water activity (a_w) of the product

The water activity of the sample was measured using a water activity meter (Novasina Labmaster), the measurements were made with duplicate samples.

2.2.4 Different methods of heat treatment of peanuts and the black seed mix

1. 4g of ground peanut was mixed with 4 g of treacle, and the mixture was heated at 180°C for different times (0, 2, 3, 4, and 5 min).
2. All components of the black seed mix were mixed without heat treatment; this mixture was used as a control.
3. The black seed mix was heated at 150°C for 10 minutes as described in Section 2.2.1
4. Peanuts and sesame seeds were boiled in water for 30 minutes before being added to a black seed mix; the black seed mix was then made as described in section 2.2.1

2.2.5 Protein extraction from the black seed mix

According to Rizzello *et al.* (2009), 3 g of the black seed mix was homogenised with 3 g of distilled water, and 30 ml of boiling distilled water was added. The sample was incubated at room temperature for 10 min and centrifuged at 14,000 x g for 20 min. The

sample was defatted using hexane, the supernatants were then filtered, and the soluble protein content was determined using the Bradford assay as described in Section 2.2.5.

2.2.6 Determination of soluble protein

A colorimetric protein assay was used according to Bradford (Walker, 2002) to determine the concentration of soluble protein. Bovine serum albumin (BSA, Sigma) was used to prepare a standard curve of the sample; 1 mg/ml of was prepared and diluted with distilled water to create a standard curve of 0.1, 0.2, 0.4, 0.6, and 1 mg/ml. Then, 100, 200, 600, and 1,000 µl of the sample were mixed with 900, 600, and 400 µl of distilled water. 3 ml of the Bradford reagent (Bio-rad) was added to 100 µl of the sample solution. The sample was incubated for 20 minutes at room temperature. The blue colour was measured at 595 nm. Measurements were in triplicate.

2.2.7 Determination of insoluble protein

A solid pellet of the protein sample was measured by the Kjeldahl method in triplicate as described in Section 2.2.2.9.

2.2.8 Determination of the glycation degree of proteins

To determine the free amino groups in the solutions the *o*-phthaldialdehyde (OPA) method was used as described by Gu *et al;* (2009). The OPA reagent was prepared by dissolving 40 mg of OPA in 1 ml of methanol. 1.91 g of di-sodium tetraborate decahydrate and 0.05 g of SDS was dissolved in 40 ml of distilled water. The volume of the solution was brought to 50 ml with distilled water and 2.35 ml of 2-mercaptoethanol was added to the solution. In quartz cuvettes, a sample of 100 µl was added to 1.8 ml of OPA reagent and a measurement was taken immediately (A0) and after standing for 5 minutes (At) the sample was allowed to stand for 5 min at room temperature. The absorbance at 340 nm was

measured using a spectrophotometer (Thermospectronic, USA). Measurements were done in triplicate.

The glycation degree (GD) was calculated as follows:

GD% = (A0 – At / A0) x 100

Where At, = absorbance of the sample after 5 minutes and A0 = absorbance of the control.

2.2.9 Measurement of stages of Maillard reaction products

Methods described by Michalska *et al*. (2008) were followed to measure different stages of Maillard reaction products by either fluorescence or absorbance. The soluble protein extracts (section 2.2.5) of black seed mix or components heat treated by different procedures (section 2.2.4) were each diluted with distilled water to final concentration of 2 mg/ml. Different stages of Maillard reaction products were measured as follows:

1. First-stage reaction products: absorbance at 305 nm.
2. Final-stage or third-stage products: absorbance at 420 nm
3. Second-stage or intermediate-stage products: FAST index: ratio of fluorescent intermediary compounds (FIC) and soluble tryptophan:

    a. The FIC was measured at λ_{EX} = 353 nm and λ_{EM} = 438 nm.

    b. TRP_{FL} (tryptophan fluorescence) was measured at λ_{EX} = 290 nm and λ_{EM} = 340 nm, which is a measure of soluble protein concentration of the sample.

    c. The FAST index was calculated as follows: $FIC/TRP_{FL.}$

2.2.10 Measurement of antioxidant properties

2.2.10.1 Ferric thiocyanate (FTC) method

The test was conducted according to the method of Saha *et al.* (2004). 4 ml of the sample (Section 2.2.4) were mixed with 4 ml of ethanol and 4.1 ml of 50 mM linolenic acid solution in 99.5% ethanol; 8 ml of 0.1 M phosphate buffer (pH 7.0); and 3.9 ml of distilled water. The mixture was placed in a dark oven at 40°C for 8 days. As positive control 4 mg of BHT dissolved in 4 ml water was added to the reaction mixture instead of the sample. Oxidation was measured after different periods of incubation. 1 ml of the sample was added to 50 µl of a mixture of 2.35 ml of 75% ethanol, 50 µl of 30% ammonium thiocyanate, and 50 µl of 20 Mm ferrous chloride solutions. The sample was allowed to stand for three minutes; after the ferrous chloride was added to the sample, the absorbance was measured at 500 nm by a spectrophotometer. These steps were repeated every 24 h for 8 days. A mixture containing hydroxy toluene (BHT) was used as a positive standard and the control was incubated with linoleic acid but without the MRP samples. Measurements were done in triplicate.

% Inhibition is calculated as:

[1 - absorbance of sample at 500 nm)/ (absorbance of control at 500 nm)] X 100.

2.2.10.2 Thiobarbituric acid (TBA) method

The TBA method was employed according to Saha *et al.* (2004). 1 ml of the sample prepared from the FTC assay (after 8 days) was added to 2 ml of 0.67% 2-thiobarbituric acid and 2 ml of 20% trichloroactic acid. The mixture was boiled for 10 minutes, and then a sample was centrifuged at 3,000 rpm for 20 min. The absorbance of the supernatant at 532 nm was measured using a spectrophotometer.

2.2.10.3 DPPH radical scavenging activity assay

The DPPH radical scavenging activity assay was performed according to Singh *et al.* (2002). 1 ml of sample was dissolved in 4 ml of water, then 1ml of dissolve sample was

added to 1ml of methanol; this was mixed with 500 µl of 0.1 mM of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). For the negative control, 1 ml of water was used in the reaction mixture instead of the sample. After 30 min of incubation at room temperature, the sample was measured at 517 nm. As positive control, 20 mg/ml of the Vitamin C (Ascorbic acid) was used as a positive control.

The percentage radical scavenging activity was calculated in following equation:

(A517sample/A517 control) x100

Where A517 control is the absorbance of sample without DPPH and A517sample is absorbance of sample plus DPPH.

2.2.10.4 Ferric reducing antioxidant potential assay (FRAP)

The FRAP assay was done according to Benzie and Strain (1996) 1 ml of sample was dissolved in 4 ml of water, The stock solutions included 300mM acetate buffer (3.1 g Sodium acetate and 16ml acetic acid), pH 3.6, 10mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40mM HCl, and 20mM $FeCl_3$ solution. The fresh working solution was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ solution, and 2.5ml $FeCl_3$ solution and then warmed at 37° C before using. 150 µl of dissolved sample were allowed to react with 2.850 ml of the FRAP solution for 30 min in the dark. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. 20mg/ml Vitamin C (Ascorbic acid) was used as a positive control and distilled water was the blank.

The absorbance of the samples was compared to a $FeSO_4$ standard curve and the FRAP values were expressed as Ferrous Equivalent (FE), the concentration of extract or chemical which gives the same absorbance as ferrous ion equivalents.

2.2.11 Addition of sodium bisulphite and ascorbic acid to the black seed mix

- The different heating procedures for the black seed mix was (described in Section 2.2.4)
- Sodium bisulphite or ascorbic acid (50, 100 and 200mg) was added to 40g of black seed mix before the final stage of roasting.

2.2.12 Enzyme-linked immunosorbent assay (ELISA)

Preparation of protein samples and antibody solutions

The concentration of soluble protein in filtered water extract (Section 2.2.4) was determined using the Bradford assay (Section 2.2.6). The protein concentrations were then adjusted with PBS to reach a concentration of 1 mg/ml.The dilutions of specific IgE sera (peanut allergy human serum and individual human serum) for the purpose of ELISA were 200 µl of serum sample to 8 ml blocking buffer 2 The dilution of secondary antibody was 1 µl of secondary antibody (alkaline phosphatase conjugated goat anti human IgG) was added to 10 ml of PBS

Proteolytic digestion of samples

The concentration of soluble protein in filtered water extract (Section 2.2.4) was determined using the Bradford assay (Section 2.2.6). The protein concentrations were then adjusted with PBS to reach a concentration of 1 mg/ml. The pH of 20 ml of each sample was adjusted to 8.4 with 1 mM NaoH. Trypsin (0.2 mg) was added to each sample, vortexed and incubated for 3 hours at 37 °C followed by heat treatment for 5 minutes at 80 °C to inactivate the enzyme and cooling on ice (Cabanillas *et al*,. 2012).

Preparation of ELISA plate

The enzyme-linked immunosorbent assay was done according to Van de Lagemaat *et al*. (2007). 100 µl of the antigen solution was added to each of marked wells of the microtitre

plates and incubated overnight at 4°C. Each sample was added to 4 wells to enable quadruplicate measurements. The plate was washed three times with phosphate buffered saline–tween (PBS-T) and blotted on paper towels after the last wash. The marked wells were incubated with 200 µl bovine serum albumin (BSA; 3 ml in 20 ml PBS) at 37°C for 1 h before being washed three times with wash buffer. Then 200 µl of diluted IgE antibody was added and incubated at 37°C for 1 h or overnight at 4°C. The plates were washed again three times to remove excess antibody. Then 200 µl of secondary antibody were added to each well and incubated at 37°C for 1 h. The plates were further washed three to five times to remove all the secondary antibody. Finally, 100 µl of alkaline phosphatase was added to each well. The estimated incubation times for the enzyme-substrate reaction ranged from 1-15 minutes. 50 µL of 3 M NaOH was used as a stop solution. After the enzymatic reaction was stopped, the plates were read at 450 nm. The measurements were made with 4 time samples.

2.2.13 SDS-PAGE and Western blot analysis

Preparation of samples for SDS-PAGE and Western blot

The insoluble proteins in the pellet after centrifugation were processed with a Ready Prep protein extraction kit (Total Protein, Bio-rad, 163-2086) by adding 1 ml of 2-D rehydration sample buffer to 0.1 g of each pellet sample in a micro-centrifuge tube. In the fume hood, 10 µl of tributylphosphine (TBP) was added, and the sample was mixed for 3 min. Then the sample was centrifuged for 30 minutes at 16 000g, the supernatant was transferred to a clean tube and used for SDS-PAGE and the Western blot method (Bio-rad Laboratories, 2006).

All of the sample protein concentrations were determined by the Bradford assay (Section 2.2.6) for soluble protein and Kjeldahl (Section 2.2.2.9) for insoluble protein to get the same amount of proteins in all samples. The protein concentration in samples was

adjusted to the same concentrations with rehydration sample buffer. Peanut protein was used as the control sample. Standard. Samples were then dissolved in SDS-PAGE sample buffer (1:1) with or without mercapto ethanol for non-reducing and reducing conditions, then, the sample was incubated at 95ºC for 10 minutes; the samples were was then put in ice for 2 min and 20 µl of each sample was loaded into the gel.

Preparation SDS-PAGE and Western blot analysis

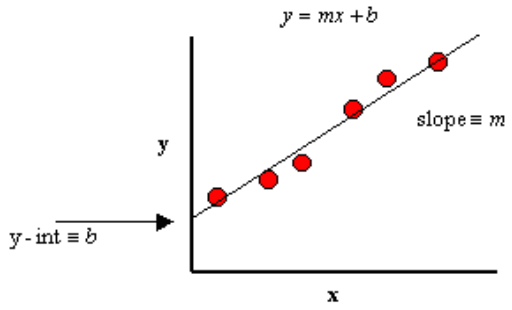
To conduct SDS PAGE, gradient polyacrylamide gel of 15-20% containing SDS was used**.** The Western blot method was done according to Cabanillas *et al*, 2012.The gel was blotted and transferred in PVDF membranes using Iblot-Invitrogen for 7 min. The membrane was incubated in the blocking buffer [bovine serum albumin BSA; 0.1 g + 10 ml of washing buffer (PBS+ tween)] for 1 hr at room temperature. The membrane was washed three times in washing buffer (TBS+ tween). Allergic patient serum (1-10 µl serum/ml blocking buffer) dilution was incubated overnight at 4ºC. The membrane was washed with washing buffer three times and then incubated at room temperature for 1 hr in secondary antibody dilution. After several washes, the membrane was incubated with SIGMA*FAST* BCIP/NBT dilution for 5-10 min.

2.2.14 Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA). Means were compared using Fisher's least significant difference test (Tukey). The calculations were performed using Sigmastat. A *P* value of 0.05 was considered statistically significant.

Standard calibration curves were calculated for the ferric reducing activity assay (FRAP) in (section 2.2.10.4) and Bradford assay to determine soluble protein content in (section 2.2.6). A calibration curve is a general method for determining the concentration of a

substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration. From two sets of data: x represents known concentrations of standards (protein or ferrous sulphate) and y represents the respective absorbance at specific wavelengths, a linear relationship between the variables x and y are plotted and a "best-fit" straight line are drawn through the data using Excell. This relationship is described by the equation = y = mx + b and is calculated using an excel spreadsheet, where **m** is the slope and **b** equals the **y-intercept,** which are shown in the figure below.



The y-intercept is the r-squared value which is the square of the correlation coefficient. The correlation coefficient is a measure of the reliability of the linear relationship between the *x* and *y* values (Values close to 1 indicate excellent linear reliability) (Clemson University, 2000).

CHAPTER 3

Development of the Black Seed Mix; Compositional and Nutritional Analysis

3.1 Introduction

3.1.1 Aims of this chapter

The aim of the study reported in this chapter is to develop a nutritional confectionary product containing seeds, nuts, and treacle with raw materials that are readily available in Middle Eastern and African countries. The composition and nutritional properties of the seed mix is inspired by an old Egyptian recipe called *Mofataka.* Whereas the purpose of the traditional recipe was to fatten girls, the aim in this study was to reformulate the recipe to provide a supplementary food with adequate amount of protein. In this study the new product is call Black Seed Mix.

The approach followed was to gain information in published literature of the proximate composition, of each of the components of the original recipe. Based on information of basic human nutritional requirements for each age group obtained from literature, a new recipe was formulated to optimise the nutritional content of the Black Seed Mix within the limits of taste and texture. The nutritional content of the recipe was calculated using an algorithm created in excel to calculate the contribution of fat, protein, carbohydrate, water and ash of each ingredient.

The Black Seed Mix made in practice was then analysed for proximate composition, amino acid profile, fats and fatty acid profile and minerals. The results for analysis were compared with the theoretical calculation of composition based on data obtained from literate. Results of analyses were also compared to basic daily nutritional requirements for each age group.

3.2 Principles of methods used in this chapter

3.2.1 Recipe development

Nutrient databases are used by food manufacturers to determine the nutrient content of their products for food labels. Food service managers plan menus for schools, hospitals,

and other institutions based on their nutrient content. A nutrient database usually contains values obtained from chemical analysis of the food. In the case where analytical data such as mineral or vitamin content of ingredients are not available, expensive analytical procedures are required (Schakel *et al.,* 1997).

The approach followed in the present study was to gain information available in the literature of the components of each ingredient to be used in the Black Seed Mix recipe such as proximate composition. Using this information, the nutritional content of the recipe was calculated using an algorithm created in excel to calculate the contribution of fat, protein, carbohydrate, water and ash of each ingredient. Based on information of basic human nutritional requirements for each age group obtained from literature, a recipe was formulated to optimise the nutritional content of the Black Seed Mix within the limits of taste and texture.

3.2.2 Proximate analysis

A quantitative method in determining the different macronutrients is the proximate analysis or the crude analysis for nutrients of a food product or ingredient. This is basically the division of feed compounds with the use of basic chemical properties into five classifications. Dry matter and ash, as well crude protein, crude fibre, and crude fat are all contained in the analysis.

The proximate analysis (crude nutrient analysis) of a food product or ingredient is a quantitative method to determine different macronutrients. Basically it is the partition of feed compounds into five categories by means of common chemical properties. The analysis contain: dry matter, ash, crude protein, crude fibre and crude fat (Babayan *et al*., 1978).

3.3 Nutritional elements in the human diet

There must be vitamins and minerals, together with proteins, fats, and carbohydrates included in the daily food intake (Smolin *et al*., 2008) if health is to be maintained. Because vitamins and minerals are only required in little amounts in food, they are referred to as micronutrients. Energy is supplied to the body by fat and carbohydrates. Growth and conservation of body structures, as well as body metabolism regulation depend heavily on protein which can also be utilised in providing energy (Smolin *et al*., 2008).

Proteins and amino acids

Proteins can be described as intricate, organic compounds made up of hundreds of amino acids and which are very essential in the maintenance of living muscle tissue. Proteins are the nutrition's building blocks for the simple reason that the digestive enzymes break them down into amino acids (Ory, 1986). Proteins have twenty two amino acids within them. The human body creates fourteen of these amino acids, provided that enough levels of essential antecedents are present in the diets taken. Eight of these amino acids are impossible for the human body to synthesise which implies that they must be derived from food, and these are known as essential amino acids which means that they must be made available in the diet for growth and preservation of health (Nakai, 1996). However, leucine and isoleucine, as well as valine and lysine, are all essential amino acids. Other types of amino acids include threonine and tryptophan, together with methionine, phenylalanine, and histidine. On the other hand, alanine, asparagine and aspartic acid and glutamic acid, as well as arginine and cysteine, together with glutamine, glycine, proline, serine, and tyrosine are all non-essential amino acids (Nakai, 1996).

Fats and fatty acids

Depending on what their composition and structure may be, fats can be described as solid or liquid at room temperature. There is solubility of lipids and fats in organic solvents but

their solubility in aqueous solvents is very slight. The absorption of Vitamins A, D, E, and K is only possible when their present in the diet make them fat-soluble (Davis and Saltos, 1996). Fatty acids (FAs) can be described as chains of hydrocarbons having a methyl group at one end known as the methyl end and a carboxyl group at the other end known as the carboxyl end. There are between 12 and 22 carbon atoms present in the common FAs present in food. FAs are classified into two major categories of saturated FAs (SFA) possessing no carbon-carbon (C-C) double bonds and unsaturated FAs which possess double bonds and having 1 to 6 C-C. Unsaturated FAs are further classified into two groups of mono- (MUFAs) and polyunsaturated fatty acids (PUFAs). There is only one C-C double bond in MUFAs while 2 to 6 C-C double bonds are present in PUFAs. Usually, the numbers of C-C double bonds are not PUFAs major focus, but rather where the placement of the double bonds is made from the methyl end. It is as a result of this that led to the creation of the two major families of PUFAs. These are categorised as omega-3 FAs (n-3 FAs) and omega-6 FAs (n-6 FAs). The first double bond in -3 FAs like linolenic acid lies on the third carbon position and the first double bond in the n-6 FAs like linoleic acid lies in the sixth carbon position from the methyl end (Kris-Etherton *et al.,* 2009). The human body is incapable of producing essential fatty acids, such as omega-3 and omega 6 ,and these need to be obtained from the right diet for growth and for the development of the brain (Davis and Saltos, 1996). All these fatty acids are not present as a structural part of cell membranes as they make important contributions in cell signalling. In addition, a regular and measured consumption of essential fatty acids reduces the risks of coronary heart disease (Sonnenberg *et al* 1996).

Saturated fats are needed by the body for energy, hormone production and building and maintenance of cellular membranes (Kris-Etherton *et al.,* 2009) and they are considered to be non-essential fatty acids. Although trans fatty acids are an unsaturated

fat, they are, nonetheless, non-essential fatty acids and should not be consumed like saturated fats as findings have identified that their intake is proportional to increase in the risk of contracting coronary heart disease (Kris-Etherton *et al.,* 2009). It can as well serve as supplement when used as development enhancements. At the present, they are non-essential fatty acids and categorised simply as such considering the fact that the human body is capable of synthesising them from other nutrients such as carbohydrates, together with other unsaturated fatty acids (Kris-Etherton *et al.,* 2009).

Carbohydrates and sugars

There are three major division groups of carbohydrates based on their chemical structure. These are sugars, oligosaccharides, and polysaccharides (FAO/WHO, 1997). Sugars are composed of monosaccharides that have single monomeric unit or one single sugar in the form of glucose, fructose, or galactose and disaccharides which have two monomeric units in the form of sucrose and lactose, as well as maltose and trehalose. Oligosaccharides consist of chains of 3 to 9 bonded to each other by glycosidic bonds. Polysaccharides, besides having 10 or more monomeric units, are represented in the diet by starch and dietary fibre (Wheeler and Pi-Sunyer,,2008). Starch is made up of numerous glucose monomers which are connected to different arrangements, forming amylose and amylopectin. These ratios are what actually determine what the starch gelation characteristics are. Globally, the principal carbohydrate in diets is starch the major storage carbohydrate and energy source of crops (Cummings and Stephen 2007).

Dietary fibre

Dietary fibre is defined by the Scientific Advisory Committee on Nutrition (SACN) as all carbohydrates that have a degree of polymerisation of three or more monomeric units (as well as lignin), that are not digested in the small intestine (Scientific Advisory Committee on Nutrition, 2015).

Dietary fibre, on the basis of solubility in water can be classified into soluble dietary fibre and insoluble dietary fibre. A good example of soluble dietary fibre is Pectin derived from apples. Dietary fibre demonstrates positive effects in controlling sugar level, specifically as a result of increased viscosity in luminal contents (Galisteo *et al*., 2008). They assist in the promotion of faecal bulking, softening, and laxation (Galisteo *et al*., 2008).

Vitamins and Minerals

Vitamins and minerals which are dubbed micronutrients have very crucial roles to play in the human body. The recommended daily allowance (RDI) for minerals are: zinc is 8-15 mg/day, iron 14-19 mg/day, magnesium 200- 400 mg/day, phosphoris -800 mg/day and calcium -800 mg/day. The RDI for vitamins are: vitamin A -800 µg/day, vitamin D- 5 µg/day, vitamin E -10 mg/day, vitamin C -60 mg/day, thiamine -1.4 mg/day, riboflavin -1.6 mg/day, niacin -18 mg/day, vitamin B6 -2 mg/day, folic acid -200 µg/day, vitamin B12- 1 µg/day, vitamin K- 80 µg/day, biotin -0.15 mg/day and panthothenic acid -6 mg/day (Story and Stang, 2005).

Calcium and vitamin D, together with phosphorous and magnesium are very essential in bone formation and body maintenance as well as in osteoporosis prevention. Calcium and vitamin K are also very important elements in blood clotting (Ervin *et al*., 2004a). Energy metabolism needs phosphorous and magnesium, together with the B vitamins. Sufficient amounts of sodium and potassium, as well as calcium, phosphorous, and magnesium are required in the maintenance of normal neural transmission and muscular function, as well as vasoconstriction and vasodilation, acid-base balance, regulation of water, and osmotic pressure (Ervin *et al*., 2004a). It is important that sufficient amount of these micronutrients be taken in order for the body to function normally. In spite of the importance of sodium, increased dietary levels of sodium carries a high risk of elevated blood pressure which in turn increases the risk of developing

cardiovascular and renal illnesses. The B vitamins and phosphorous, together with magnesium, copper, and zinc are responsible for regulating enzyme activities. Zinc, in addition, is quite vital for the structure of protein, while copper, along with vitamins C and E aid in the protection of the body against oxidative stress (Ervin *et al*., 2004b). Vitamins B6 and B12, together with folate are very important when it comes to cognitive functioning, specifically in adults. They are essential when it comes to pregnant women in order that defects of the neural tube in the emerging foetus are prevented (Ervin *et al*., 2004b). Vitamin A is also important in pregnant women and in the emerging foetus, because of its importance in the development of the embryo, growth and immunity, and possessing of normal vision (Ervin *et al*., 2004b). The risks for certain cancers and cardiovascular disease can be potentially lowered by β-carotene derived from vitamin A. β-carotene, along with vitamins C and E can also reduce the risk of cataracts associated with aged related macular degeneration (Ervin *et al*., 2004b).

3.4 The black seed mix: traditional use and traditional compositions

Mofataka is a well-known traditional Egyptian recipe. It is a type of jam that has been used in some Arab countries for decades to improve weight gain (Figures 3.1 and 3.2). Women's beauty was measured by weight and height, and slim women were not desired or wanted at that time. Strong women were needed for the hard work they used to do, reflected in a proverb: "A man may work from sun to sun, but woman's work is never done". Additionally, women who were large and fat were thought to be healthy and to live in luxury.

Figure 3.1. Mofataka jam (4women.co, 2012) **Figure 3.2.** Mofataka (Mohammed, 2012)

As a result of these beliefs, most women were eager to gain more weight, and to them, *Mofataka* was the answer. There are many ways to make *Mofataka*, but the main ingredients are fenugreek, sesame, flour, oil or margarine, black seeds, and treacle (sugarcane honey). No research has been done on *Mofataka* or on its nutritional value. The recipe has a strong aroma and bitter taste because of the amount of fenugreek used and the black seeds. The purpose of this project is to adjust the ingredients of the recipe so that it could serve as a high-protein nutritional food supplement. I considered adding more protein, reducing the fat, and overcoming the unpleasant aroma and bitterness without ignoring the need for minerals and vitamins.

3.5 Literature review of the nutritional composition of the components of the black seed mix developed in this project

Figure 3.3. Components of the black seed mix developed in this project

3.5.1 Black seeds (Nigella sativa)

Nigella (*Nigella sativa* L.) is an annual herbaceous plant related to the *Ranunculaceae* family (Al-Gaby, 1998) that grows in countries such as India, North Africa, and those around the Mediterranean Sea (Saad and Said, 2011). Nigella seeds have little odour, but when ground, they develop a spicy scent; the taste is aromatic and slightly bitter. Figure 3.4 shows its dark grey or black colour and its small (1–5 mm), angular shape. These seeds are originally white when they are in their fruit capsule; they turn black when they are exposed to air (Figure 3.5) (Saad and Said, 2011).

Figure 3.4. Nigella seeds

Figure 3.5. Nigella flower

Nigella sativa is a seed which is rich in nutrients. Whole mature Nigella seeds proximate analysis in Table 3.3 revealed that the range of the moisture content is from 5.52% to 7.43%, and the crude protein content is 20% whilst the range of ash content is from 3.77% to 4.92%. The carbohydrate is 25.7% and the range of ether-extractable lipid content is 34.49 % (Abdel-Aal and Attia, 1993; Salem, 2001; Takruri and Dameh, 1998; Attai, 2003). The oil extracted from Nigella seed is seen as a new source of edible oils which play vital role in the health and nutrition of humans. It is reported that Nigella seed oil possesses antitumor activity (Worthen, Ghosheh *et al*., 1998) and antioxidant activity (Burits and Bucar, 2000). It also possesses anti-inflammatory activity (Houghton *et al*., 1995) and antibacterial activity (Morsi, 2000), and also has effect with regards to the immune system (Salem and Hossain, 2000). Linoleic acid which ranges from 50.3–49.2% is the main unsaturated fatty acids. This is followed by oleic acid which ranges from 25.0–23.7%. However, palmitic acid, ranging from17.2–18.4% is the major saturated fatty acid. Among its other fatty acids s are myristic (C14:0) and myristoleic (C14:1), together with palmitoleic (C16:1) and margaric (C17:0). Others are margaroleic (C17:1), stearic (C18:0), linolenic (C18:3), and arachidic(C20:0), as well as eicosenoic (C20:1), behenic (C22:0), and lignoceric acids (C24:0) (Al-Jassir, 1992 and Cheikh-Rouhou *at el*, 2007). A significant amount of essential minerals are present in Nigella seeds. In the black seeds, the most abundant element is potassium. The next most abundant elements are phosphorus and sodium respectively. The other elements in their order of abundance are Cu, Mn, Zn, Fe, Ca, and Mg.

Traditional use

The black seed can be named a “miracle herb”. For thousands of years, it was considered capable of providing an outstanding cure for numerous diseases caused by bacteria or

viruses (Goreja, 2003). It has a combination of over 100 effective components that grant the black seed amazing strength to support the body's immunity and increase the body's nature in a manner that no single material can do (Goreja, 2003). *Nigella sativa* seeds have been used for a long time for food and medication in many countries, including Egypt, Syria, Iran, India, China, and Tunisia. They are added to bread and to some food recipes as a spice (Aboutabl, *et al*., 1986). They are also used in the making of some traditional sweet dishes and in flavouring foods, especially bakery products and cheese.

For a long time, ancient Egyptians and Greeks used Nigella for nasal congestion, toothache, and menstrual discomfort and to increase milk production. It has also been used for a long time in traditional Islamic medications and in India and China for the prevention and healing of a large number of illnesses, such as asthma, obesity, dysentery, infections, back pain, headache, hypertension, and gastrointestinal problems (Saad and Said, 2011). The prophet Mohamed (PBUH) said, "Black seeds can cure all diseases except death". Furthermore, in a well-known book, *The Canon of Medicine*, the researcher Avicenna wrote that the seeds restore the body's energy and help to quickly heal dispiritedness. The seeds' oil has also been used to cure skin conditions such as eczema and boils (Saad and Said, 2011).

3.5.2 Fenugreek seed (Trigonella foenum-graecum)

Fenugreek (*Trigonella foenum-graecum* L.) is an herbal plant that grows as a self-pollinating crop. It was first found in the Indian subcontinent and the eastern Mediterranean region. It later extended into central Asia and North Africa, and more recently, it has been grown in central Europe, the United Kingdom, and North America (Tapan and Srichamroen, 2010). It is related to the Papilionaceae section of the Leguminosae family. The species name foenum-graecum means "Greek hay", while the genus name *trigonella* means "little triangle"; it took its name from its leaflets' triangle

shape. Figure 3.6 shows that fenugreek plants grow to a height of up to 0.5-0.8 mm and are tap rooted, with one main stem. The plant stem has branches up to 0.4 mm long with trifoliate leaves that are 2-3 cm long. Flowers are a cream colour and measure 1 cm in length. They occur in long, slender 15 cm green pods that turn yellow-brown. Each mature brown pod contains approximately 20 small yellow to brownish-yellow seeds (Figure 3.6).

Figure 3.6. Fenugreek plant and seeds

The chemical composition of fenugreek seed in table 3.3, the lipids content was reported to be 7%, 44% carbohydrates, 30% of protein, 3.3% ash and 5% moisture (El Nasri and El Tinay, 2007). Fenugreek contains the vitamins ascorbic acid, β-carotene, nicotinic acid, inositol, A, D, and is rich in phosphates and lecithin. It contains considerable quantities of iron in a readily absorbable form. It also contains other minerals, such as Ca, Zn, Mg, and K (Abdel-Nabey and Damir, 1990; Akbari *et al* 2012). The fenugreek seeds are rich in leucine, valine, lysine, threonine and phenylalanine (Table 3.7 and Table 3.8) (Akbari *et al* 2012). Fenugreek is reported to contain the amines trimethylamine and betain which are reported to stimulate the appetite because of their effect on the nervous system (Grieve, 1971). It also contains minute quantities of essential oils, with 40 different compounds, including alkanes, sesquiterpenes, and lactones

(Parthasarathy *et al*., 2008). Fenugreek is a type of seed with a high content of hemiterpenoid y- γ-lactone and sotolone (3-hydroxy 4, 5-dimethyl 2(5H)-furanone). Sotolone is a spicy flavour and can be found in a sauce used by the brand Maggi. The seed also contain some aromatic constituents such as n-alkanes, sesquiterpenes and nonalactone. They have been found also to be rich in saponins. Three steroidal saponins, namely diosgein, gitogenin, and tigogenin, have been reported to be present in fenugreek seeds (Tapan and Srichamroen, 2010).

There are three active component groups – 4-hydroxy-isoleucine, galactomanans and steroidal saponins – believed to facilitate the potential health benefits of fenugreek. Cough and mucus can be loosened with the use of fenugreek, making them possible to be expelled from the body. This may account to its use in situations of bronchitis and cough, as well as in congestion Diabetes. Fenugreek seeds have demonstrated their ability in partial alleviation of type-1 and type-2 diabetes (Akbari *et al,* 2012). Hypoglycaemic: - Fenugreek seeds have also demonstrated their ability to aid in the lowering of blood sugar. A lot of experiments have been conducted to examine the possible fenugreek anti-diabetic effect. All experiments demonstrated fenugreek seeds' ability in the reduction of blood glucose levels and improvement of glucose tolerance. One study required 15 grams of powdered fenugreek seed to be added in the meal of individuals suffering from type-2 diabetes. The result was that the rise in their post-meal blood glucose went down. Another study was able to discover that a twice daily intake of 2.5 grams of fenugreek for three months resulted in the lowering of the blood sugar levels in individuals suffering from mild type-2 diabetes but nor the severe one (Akbari *et al*, 2012). The existence of phenol and saponins in fenugreek is reportedly linked to the fenugreek antioxidant benefits (Akbari *et al*, 2012).

Traditional use

In Egypt, the seeds' plant is called *helba*. It is traditionally used in preventing fevers and settling the stomach and has been utilised for diabetes. The seeds are soaked in water and then allowed to sprout. After growing to about two to three inches high, the green part is eaten raw with the seeds (Tapan and Srichamroen, 2010). In India, the seed powder is used as a spice in curry. The seeds can be an inexpensive source of high-quality protein (El Nasri and El Tinay, 2007). It was noted that adding fenugreek flour to wheat flour at a ratio of 10% makes it rich in protein, fibre, total calcium, and total iron (El Nasri and El Tinay, 2007).

A review by Basu and Srichamrone, (2010) reports traditional uses for fenugreek. It is an excellent in-house treatment for heartburn and acid reflux disease. It contains mucilage, a gluey substance that can cover and protect the lining of the stomach and gastrointestinal tract. The advantages of this plant are that it is cheap and readily available, and can also be taken by children, especially when its bitter taste is disguised.

Fenugreek seeds are also used as ground flower also has ground seeds, which are mainly used to add flavour in animal feeds. As a result of its powerful odour of coumarin, the powder is used to flavour cattle forage, as well as making damaged hay tasty to cattle (Tapan and Srichamroen, 2010). In the United States, the powder is also used in different industries including tobacco and food industries (Tapan and Srichamroen, 2010). Fenugreek seeds can also be used for the skin in cases of minor burns and for skin conditions such as eczema and boils. The seed flour is used to make a thick paste that is spread on problem skin areas. It is known to reduce fever and relieve sore throats, and it can alleviate dandruff from the scalp and stop hair loss, especially in men (Akbari *et al*, 2012). Women can also find some usefulness in fenugreek, such as the inducement of childbirth, relief from menstrual pains, and for lactation. In addition, men can use

fenugreek for conditions such as premature ejaculation and decreased libido. Fenugreek is therefore one of those rare natural products with numerous health benefits for both men and women (Basu and Srichamrone, 2010).

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3.5.3 Sesame seed (Sesamum indicum L.)

Sesame (*Sesamum indicum* L.) is an annual herbal plant that is part of the genus Sesamum, which belongs to the Pedaliaceae family. This family contains about 20 species (Bedigian, 2010). Sesame is a plant that is mainly grown in China, Russia, and the United States because it is rich in protein. Research shows that the plant perform well in tropical and subtropical climate, but more adapted to more cold conditions like those found in Russia, China, and the United States (Kuol, 2004). In addition to being rich in protein, its oil is also rich in unsaturated fatty acids. Research shows that substantial amount of the world produce of sesame seeds is utilized as oil (Elleuch *et al*., 2007). As a result of being highly nutritious, countries such as Japan and China have used the plant to prevent many diseases for several thousand years (Chen *et al.,* 2005).

It is an annual self-growing plant. Figure 3.7 shows that sesame is generally characterised by oval-shaped green leaves. The plant also produces flowers, which are white or pink bell-shaped. These later turn into fruit capsules. The flowers are tubular in shape, 2-2.5 cm long, and white, though some have pink or purple markings. The seeds come in a variety of colours, including shades of brown, brick red, black, yellow, beige, grey, and white, depending on the cultivar (Figure 3.8) (Elleuch *et al*., 2007)

Figure 3.7. Sesame seeds

Figure 3.8. Sesame plant

In Table 3.3, the displayed chemical composition of sesame indicates that the composition of the seed is 44% fat, 25% protein, 13.5% carbohydrate, 12% fibre, and 5% ash (Elleuch *et al*., 2007; Latif and Anwar, 2011; Shyu and Hwang, 2002). The composition of the major protein has 67.3% of globulins and 8.6%, together with 1.4% of prolamine and 7% of glutelin (Latif and Anwar, 2011). Protein from sesame is very high in methionine at 3.2%, which is quite uncommon for most plant proteins. All these unique properties are what make sesame seed to be an extraordinary protein source and as supplement for soybean and peanut, together with other vegetable proteins that do not contain enough methionine, in upgrading their nutritive qualities (Latif and Anwar, 2011). Carbohydrates present in sesame seed are found to have 3.2% of glucose and 2.6% of fructose, together with 0.2% of sucrose while the rest of the content is dietary fibres (Anilakumar *et al*., 2010). Sesame oil, in comparison with other cooking oils, has high resistance to oxidation. The research conducted by Namiki (1995) identified antioxidative compounds such as lignans and phenols present in sesame seed oil. Triglycerides which contain 40% of singly unsaturated oleic acid and 45% of doubly unsaturated linoleic acid, together with approximately 10% of saturated fats are what sesame oil is generally composed of. The storage life of sesame oil is very good due to the fact that it has powerful antioxidant properties while triply unsaturated fatty acids are absent. A distinctive flavour

obtained from a good number of compounds is present in oriental sesame. Such flavour is only formalised when the process of roasting is taking place (Kouol, 2004).

Traditional use

The use of sesame oil goes back deep in history, perhaps as far back as 2000 BC. It may be one of the oldest oils used (Abd El-Himed and El-Bramawy, 2011). In the Orient, sesame was long considered a food that was good for health, one that intensified energy and fought ageing (Namiki, 1995). In Saudi Arabia and in all other Middle Eastern countries, sesame seeds are used as a spice and decorative substance. They are added to food, bread, and pastries. There is a traditional well-known sweet called *halawa* that is made from its seeds and is eaten at breakfast. There is also a salad dressing called *tahinah* that can be used as a sauce to make very delicious food (Abou-Gharbia *et al* 2000; Abu-Jdayil and Asoud, 2002).

3.5.4 Millet

Millet is the name given to a number of small-grained annual cereal grasses that include several different species (Figure 3.9). The best-known types are pearl, finger, proso, and foxtail millets. Some other types are local to particular regions, such as kodo, barnyard, fonio, and teff millets (Abd El-Himed and El-Bramawy, 2011). The seeds are usually used for human consumption or for animal feed. In relation to other cereal grains, millets are generally favoured because they require less demanding growing conditions, such as low-fertility soil, high-temperature conditions, and climates with low rainfall. In addition, they require shorter growing seasons. Pearl millet (*Pennisetum glaucum* L. R. Br.) is one of the most important drought-tolerant crops of the tropical and subtropical regions of the world; it can produce good yields of grain. Most botanists believe that pearl millet was first found in Africa and later transferred to India and the rest of the world. Soon after its adoption, it

spread widely across the semi-arid tropics of Africa and Asia (Abd El-Himed and El-Bramawy, 2011).

Figure 3.9. Millet Plant

In the poorest countries of the word and among the people who live in abject poverty, pearl millet serves as the basic staple which the people depend on. Pearl millet is considered, among cereals and millets, as one of the best crops when it comes to drought resistance. It is also one of the four most important cereals of rice, maize, sorghum and millets which are cultivated in the tropics (Nambiar *et al.*, 2011). Pearl millet has carbohydrate content of 67.5%, with starch representing approximately56 to 65% and within the starch content are content amylose in the amount of 20 to 22% and free sugar in the amount of 2.6 to 2.8%. The pearl millet also contains 1.2 % fibre and 11% protein which are rich in amino acids lysine and isoleucine. Fat content of 5%, of which the fatty acids 75% content of unsaturated, is present in millet. Millet has also rich content of B-vitamins and potassium, together with phosphorous and magnesium, including iron, zinc, copper and manganese as displayed in Table 3.3 (Nambiar *et al.*, 2011). A high amount of iron (8mg/100g) and zinc (3.1mg/100g) are contained in pearl millet, and because of pearl millet's chemical composition, it offers a lot of health benefits and assists a lot in increasing the levels of haemoglobin, thereby lessening anaemia. A high amount of

antioxidants is present in millet and combined with nutrients, they may prove beneficial in holistic health and wellbeing (Nambiar *et al* 2011). The promising prevention and treatment of cardiovascular diseases and diabetes, as well as arthritis and certain cancer illnesses are highlighted in omega-3 fatty acids presence in pearl millet grain (Simopoulos, 2000). The revelation of Chandrasekara and Shahidi (2011) confirmed that kodo millet phenolic extract demonstrated higher activities of inhibition against LDL cholesterol and liposome oxidation, in contrast to that of pearl millet. They confirmed millet dehulled and hulls grains inhibition of 1 and liposome oxidation, as well as HT-29 adenocarcinoma cells proliferation.

Traditional use

Pearl millet is a primary cereal in India, Syria, Saudi Arabia, and many African countries, especially those in western and southern Africa. In Africa, it is used primarily to make traditional porridges and for making local beers and wines. In some West African countries, it is used to make couscous and fritters. It is also used to make cakes and food for young children. Malted pearl millet is also mixed with flour for making bread (Gomez and Gupta, 1993).

3.5.5 Peanut

Arachis hypogaea L. is known by many different names, including peanut, groundnut, monkey nut, goober, and earthnut. Peanut is found in the Papilionaceae subfamily, which is part of the Leguminosae family. It can be distinguished easily because it is among the few legumes that flowers above the ground while their fruits grow underground. The peanut plant has alternate and pinnate with four leaflets. It flowers are yellow and have axillary branches, but these change with climate. The leaflets are softly hairy and obviated in shape, and about 3.5 cm long (Figure 3.10). It can also be noticed because of its unique

stem and side branches. Peanuts are categorized by their growth habit. As a result, there are two categories, including upright with a straight central system, as well as large with many lateral branches (Sanders, 2003).

Figure 3.10. Peanut plant

The peanut kernel contains about 16.2-36% protein, which consists of albumin, globulins, and glutelins. While peanuts are rich in amino acids, they are notably very low in essential amino acids such as lysine, methionine, and threonine. The oil content of peanuts is between 47 and 50%, most of which is unsaturated fatty acid (80%). Oleic acid constitutes about 40-45% of the fat content; 30-35% is linoleic acid. Peanuts are considered an excellent source of mono- and polyunsaturated fatty acids. Though the oil is high in calories, it has been shown to improve cardiovascular health. Peanuts contain 18% carbohydrates, with a starch content of 0.5-5%, and a sucrose content of 4-7% Table 3.3. In addition, peanuts have a good amount of vitamins, including riboflavin, thiamine (which is destroyed if roasted), nicotinic acid, and vitamin E, with appreciable amounts of B-complex vitamins and vitamin K, but practically no vitamin A, C, or D (Woodroof, 1983).

Peanuts have demonstrated their relationships to improved cardiovascular health despite the fact that the caloric value of their oils is quite high. A high monounsaturated fatty acid (MUFA) oleic acid diet has been linked to reduction in low density lipoprotein (LDL) cholesterol while high density lipoprotein (HDL) cholesterol is increased. Compared to HDL which is regarded as 'bad' cholesterol, LDL is looked upon as 'good' cholesterol (Alper and Mattes, 2003). Alper and Mattes (2003) conducted a study which reported of improvement in cardiovascular health due to regular consumption of peanuts. Besides MUFA, peanuts can provide rich source of magnesium, fibre and folate, as well as vitamin E, copper, and arginine. All these contain risk reducing properties of cardiovascular illness (Alper and Mattes, 2003). The consumption of peanuts declined during the early 1990 due to fear of contamination with aflatoxins and because of health and dietary matters regarding the fat content of peanuts and questions surrounding peanuts link to allergenicity matters. Nonetheless, consumption of peanuts has increased with the low carbohydrate diet popularity and upon the discovery of its high satiation effect and its link to improved health (USDA, 2002).

Traditional use

Peanuts are consumed in various processed forms all over the world. They can be raw, boiled, or roasted and are used in sweets, candies, and snacks. More than one-third of the groundnuts produced are used as food on a worldwide basis. In the United States, about 50% of the peanuts grown are consumed as peanut butter. In other countries, they are used as a source of good oil (Micucci, 1997) in India and in many Asian countries, the extracted oil is a very important cooking medium.

3.5.6 Treacle of sugarcane

Treacle, also known as black honey in Egypt, is an important food for many populations. Treacle is concentrated cane juice without any sucrose removed. Treacle is usually

produced in small mills, mainly in the private sector, located near sugarcane cultivation areas (Figure 3.11). The process is carried out as a traditional food industry controlled mainly by the experience and practice of treacle makers. Sugarcane is cultivated in Africa, South and North America, and Asia (Broadhead and Zummo, 1988). It is a popular product consumed widely by a majority of the population in Egypt (Anonymous, 1993). Sugarcane is grown in tropical areas. It belongs to the grass family, which is part of the genus *Saccharum*. The primary objective of cultivating sugarcane is to obtain the largest possible yield of good-quality cane sugar and other important secondary products, mainly sugarcane syrup (Spencer, 1913). However, in Brazil, a very cheap fuel has recently been successfully developed from the cane.

Figure 3.11. Treacle of sugarcane

The desirable characteristics of sugarcane varieties grown for syrup production are the ability to produce a high yield of medium-sized to large stalks and a high percentage of extractable juice with high total soluble solid content. The ability to mature in a comparatively short growing season and resistance to disease are additional desirable characteristic (Broadhead and Zummo, 1988). According to Anonymous (1993), the major component of treacle is sucrose (75%), while glucose and fructose constitute only from 2-4%. There are small amounts of other substances, such as non-organic acid, starch, wax,

fats, and gum but low in protein (2%), although it is rich in minerals, such as potassium, calcium, phosphorus, iron, copper, sodium, and lead (Broadhead and Zummo, 1988). Treacle is characterised by its low price and high nutritive value, i.e., its high content of sugars and mineral elements, especially K, Na, Ca, Mg, P, and Fe. Molasses from sugar cane provides a ready source of plant derived compounds, including polyphenols and flavonoids (Wright *at al*., 2014).

The non-specific polyphenol content in treacle compares favourably to other rich sources of polyphenols such as coloured rice brans, raspberries, raisins and black pepper; the ORAC value of treacle is also comparable to other rich sources of antioxidants (Min *et al*., 2010; Rothwell *et al*., 2013). Many instances of evidence of polyphenols moderating carbohydrate metabolism have been demonstrated *in vitro*, acting by inhibition of glycolytic enzymes as well as inhibiting or delaying intestinal glucose uptake (Hanhineva et *al* ., 2010).

Traditional use

According to The Egyptian Organization for Standardization and Quality control ES No.356, Treacle is a heavy density sweet liquid produced by concentrating sugarcane juice with controlled heat such that the product does not have burnt flavour (Anonymous, 1993). As observed from the definition, it is clear that Treacle is produced in sugarcane producing regions of the world. Research shows that the product is usually produced by small private sugar mills. The production process is traditionally controlled by experience and practice of treacle maker (Stokes *et al*., 1961).

3.5.7 The global supply of ingredients for the black seed mix

Table 3.1 shows the global supply of ingredients for the black seed mix in 20 different countries. It shows that the supply of most ingredients would be sufficient to produce a

black seed mix in commercially viable quantities. However, the global supply of black seeds and fenugreek is limited compared to the other ingredients. The rest of the figures indicate that it could be feasible to produce a product such as the black seed mix on a large scale to potentially supply communities in need.

Table 3.1. Global supply of ingredients for the black seed mix in specific years

Black seed mix	Global supply by metric ton (MT) in specific years
Peanuts	15,709,036-230,449 (2010)
Fenugreek	110.00 (2008-2009)
Black seeds	800-700 (2000-2001)
Millet	10,940,000-261,610 (2010)
Sesame	722,900-32,000 (2010)
Sugarcane	719,157,000-9,715,430 (2010)

(Faostat.fao.org 2011).

3.6 Materials and method

The materials and methods used within this study were described in Chapter 2 (sections 2.2.1)

3.7 Results

3.7.1 Black seed mix recipe

Table 3.2 shows the percentage of each component of the Black Seed Mix recipe that was developed based on information gathered form published literature of the proximate composition of components (Table 3.3)

Table 3.2. Percentage of ingredients in the black seed mix

Ingredients	Weight (g)	%
Sesame	18	10.5
Peanuts	18	10.5
Fenugreek	10	5.8
N. sativa	5	2.9
Millet	15	8.8
Treacle	105	61.4
Total	171	99.9

Figure 3.12. A sample of the Black seed mix

Table 3.3. Proximate composition of components in Black Seed Mix derived from literature (RM) and respective contribution to proximate composition in the recipe (Prod).

Ingredients %	**%**	**% Ash**		**% Moisture**		**% Protein**		**% Lipids**		**% Carb**		**% Fibre**		
	Rec	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	
Peanut	10.50	2.90	0.30	5.00	0.52	28.50	2.99	47.30	4.96	10.00	1.05	2.80	0.29	
Fenugreek	5.80	5.00	0.29	5.00	0.29	38.00	1.34	7.00	0.40	44.00	1.75	24.00	1.39	
N Sativa	2.90	4.00	0.11	4.00	0.11	22.00	0.64	38.30	1.00	35.70	1.04	ND	ND	
Sesame	10.50	4.00	0.42	0.35	0.03	23.80	2.49	48.00	5.04	11.01	1.15	12	1.26	
Millet	8.80	2.00	0.17	7.80	0.68	6.00	0.52	3.00	0.26	58.00	5.08	2.00	0.17	
Treacle	61.40	7.00	4.29	20.00	12.28	ND	ND	ND	ND	78.25	48.04	ND	ND	
Total (%)	99.93		5.60		13.93		8.00		11.67		58.13		3.11	99.66

Rec: Recipe representing % of ingredients in the total recipe, **RM:** Raw material in the recipe; the % of ash, fat or protein, and moisture present in the raw material, **Prod**: Ash, fat, protein, or moisture in the ingredient as a % of the recipe, **ND:** Not determined.

Table 3.3 shows the theoretical analysis of the components of the Black Seed Mix that was developed. Results of proximate composition of the individual components were obtained from the literature.

3.7.2 Results of analysis of the proximate composition of the black seed mix

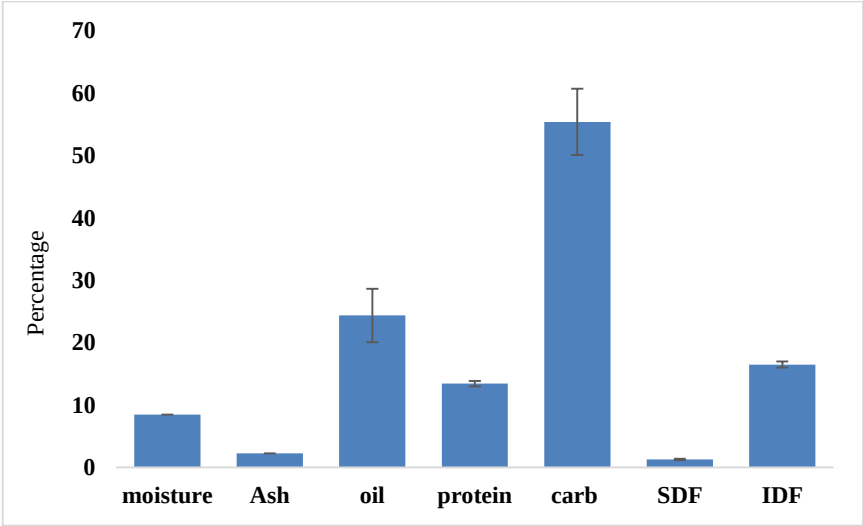

Figure 3.13. Proximate composition of the black seed mix.

The proximate analysis of the black seed mix prepared as described in materials and methods is presented in Figure 3.13. It contains 24.37% oil, 13.44% protein, 8.48% moisture, 17.30% total soluble and insoluble fibre, and 2.26% ash. The remainder is carbohydrate, which consists mainly of sugar (55.39%). The protein and fat content are higher than the theoretical value as shown in Table 3.3. This discrepancy could be due to variations found in literature of proximate analysis of the various components. The chemical composition of all ingredients was researched in the literature. A table (Table 3.3) was constructed in Microsoft Excel to combine compositions of components and to calculate the proximate composition of the end product.

3.7.3 Fatty acid composition of extracted oil

Table 3.4. Main fatty acids in 100g of oil extracted from Black seed mix.

Fatty acid	In 100g oil %	In 100g black seed mix %
Tetradecanoic acid (C14)	0.03±0.005	0.0075
Palmitic acid (C16)	6.19±0.35	1.54
Stearic acid (C18)	2.36±0.61	0.59
Oleic acid (C18:1)	20.74±1.46	5.19
Linoleic acid (C18:2)	29.07±1.6	7.25
Linolenic acid (C18:3)	0.56±0.12	0.14
Total	58.97	13.71

The black seed mix oil content averaged 24.37% (Figure 3.13) as determined by the Soxhlet extraction. An analysis of the different fatty acids by the gas chromatography method showed that the oil in the black seed mix is composed of a mixture of saturated and unsaturated fatty acids and the levels are comparable with the theoretical calculations as compiled by information from the literature (Table 3.5). It contains a high proportion of unsaturated fatty acids, namely oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and saturated fatty acids such as stearic acid (C18:0), tetradecanoic acid (C14:0) and hexadecanoic acid (C16:0) (Table 3.4). More than 44% of the fatty acids could not be characterised. An explanation could be that only one esterification process of fatty acids (2-ethyl hexanol) was used for analysis by gas chromatography the present study. Because the physical state of the lipids can vary, more than one esterification process is often required to obtain the complete spectrum of fatty acids form a food sample (Isbell et al., 2008).

The results of the analysis conducted in the present study (Table 3.4) show that the level of stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) in the black seed mix was higher than in theoretical calculations, but tetradecanoic

acid (C14:0) was a new compound reported by analysis in this study and was not found as a component of any of the components of the black seed mix reported in literature (Table 3.5). Since the concentration of linoleic acid is relatively high (7.25%), the product might be expected to be easily oxidised, which could affect the shelf life.

Table 3.5. Composition of fatty acid in published literature (RM) and related to the recipe (Prod)

Lipids	**Peanut**	**(4.96%)**	**Fenugreek**	**(0.40%)**	***N. sativa***	**(1%)**	**Sesame**	**(5.04%)**	**Millet**	**(0.26%)**	**Total prod**	**%**
Fatty acid	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**		
Myristoleic 14:1	ND	ND	ND	ND	0.10	0.001	ND	ND	20.25	0.05	0.02	
Palmitic 16:0	11.40	0.56	0.50	0.002	17.20	0.17	8.40	0.60	ND	ND	1.33	
Palmitoleic 16:1	ND	ND	ND	ND	1.15	0.01	ND	ND	ND	ND	0.01	
Stearic 18:0	2.40	0.12	2.00	0.008	2.80	0.028	5.40	0.38	51.13	0.13	0.67	
Oleic 18:1	46.80	2.32	52.60	0.21	25.00	0.25	40.70	2.93	24.42	0.06	5.77	
Linoleic 18:2	32.00	1.59	40.00	0.16	50.40	0.55	44.20	3.18	4.07	0.01	5.49	
Linoleic 18:3	ND	ND	0.60	0.002	0.34	0.003	0.40	0.03	ND	ND	0.03	
Arachidic 20:0	1.30	0.06	0.20	0.001	0.14	0.001	ND	ND	ND	ND	0.06	
Eicosenoic 20:1	1.60	0.08	0.20	0.001	0.32	0.003	0.50	0.04	ND	ND	0.12	
Behenic 22:0	3.00	0.15	0.20	0.001	2.00	0.02	0.40	0.03	ND	ND	0.20	
Lignoceric 24:0	1.50	0.07	ND	ND	ND	ND	ND	ND	ND	ND	0.07	
Total fatty acid %		4.95		0.4		1.02		5.04		0.26	13.77	

RM: Fatty acid of the component of the recipe in published literature, **Prod:** From theoretical studies of fatt acids in the recipe, **ND:** Not determined.

3.7.4 Amino acid composition of the black seed mix

Table 3.6. Amino acids in the black seed mix

Amino Group	Per 100 g black seed mix %
Essential Amino Acids	
Cysteine	0.17±0.001
Isoleucine	0.40±0.008
Leucine	0.85±0.003
Lysine	0.54±0.003
Methionine	0.19±0.003
Phenylalanine	0.55±0.007
Threonine	1.3±0.005
Valine	0.46±0.002
Total	4.46
Non-essential Amino Acids	
Tyrosine	0.41±0.004
Histidine	0.19±0.004
Arginine	1.31±0.013
Serine	0.34±0.001
Proline	0.62±0.000
Glycine	0.81±0.004
Alanine	0.57±0.000
Aspartic acid	1.28±0.041
Glutamic acid	2.61±0.027
Total	8.14

The amino acid values obtained from literature are shown in Tables 3.7 and 3.8. The levels of the amino acids of the practical analysis (Table 3.6) are similar to the theoretical values (Table 3.7), apart from lysine, which has a value of 0.54% compared to the theoretical value 0.74% (Table 3.7). This difference could be due to the Maillard reaction that the product underwent during roasting, which will be discussed in Chapter 5. The values for the nonessential amino acids also compare well with that of theoretical analysis (Table 3.8), apart from histidine that is lower than the theoretical value: 0.19

versus 0.88. The histidine content could have been reduced due to thermal treatment and/ or binding with other components in the black seed mix that reduced its extractability.

Table 3.7. Essential amino acid composition based on the literature

Amino acid	**Peanut**	**(2.99%)**	**Fenugreek**	**(1.34%)**	***N. Sativa***	**(0.64%)**	**Sesame**	**(2.49%)**	**Millet**	**(0.52%)**	**Total prod**	**%**
Essential	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**		
Cysteine	0.81	0.024	2.40	0.03	2.96	0.02	1.56	0.04	ND	ND	0.11	
Isoleucine	3.40	0.10	3.50	0.05	3.46	0.02	4.79	0.12	5.01	0.03	0.32	
Leucine	5.50	0.16	7.50	0.10	5.82	0.04	3.1	0.08	13.11	0.07	0.45	
Lysine	4.00	0.12	8.00	0.18	4.04	0.05	11.52	0.29	2.92	0.06	0.70	
Methionine	0.60	0.02	0.60	0.09	2.65	0.06	0.02	0.0004	1.70	0.09	0.26	
Phenylalanine	6.40	0.20	4.30	0.06	3.61	0.02	2.89	0.07	6.25	0.03	0.38	
Threonine	2.50	0.80	3.60	0.09	4.30	0.03	3.59	0.09	4.25	0.02	1.03	
Valine	4.00	0.12	3.50	0.05	4.61	0.03	0.49	0.01	ND	ND	0.21	
Total Amino acid %		0.78		0.41		0.18		0.93		0.19	3.46	

RM: Essential amino acid in raw material, **Prod:** Essential amino acids in the recipe, **ND:** Not determined.

Table 3.8. Nonessential amino acid composition based on the literature

Amino acid	**Peanut**	**(2.99%)**	**Fenugreek**	**(1.34%)**	***N. Sativa***	**(0.64%)**	**Sesame**	**(2.49%)**	**Millet**	**(0.52%)**	**Total % prod**
Nonessential	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	**RM**	**Prod**	
Tyrosine	4.09	0.12	3.40	0.05	3.59	0.02	3.30	0.08	2.91	0.02	0.28
Tryptophan	ND	ND	1.50	0.02	ND	ND	2.79	0.07	ND	ND	0.09
Histidine	3.17	0.09	3.10	0.04	3.35	0.02	29.30	0.72	2.62	0.01	0.88
Arginine	11.00	0.32	9.90	0.13	9.19	0.06	17.81	0.44	4.90	0.02	0.97
Aspartic acid	12.00	0.35	11.50	0.15	8.94	0.06	4.02	0.10	9.10	0.04	0.71
Glutamic acid	20.00	0.60	17.10	0.22	24.74	0.16	0.82	0.02	27.00	0.14	1.14
Serine	6.00	0.18	5.30	0.07	4.31	0.03	2.70	0.07	5.27	0.06	0.41
Proline	5.00	0.15	5.70	0.08	4.90	0.03	4.14	0.10	5.57	0.09	0.45
Glycine	7.00	0.21	5.30	0.07	5.61	0.04	4.90	0.12	3.14	0.02	0.46
Alanine	4.53	0.16	3.80	0.05	3.73	0.02	2.09	0.05	6.25	0.03	0.31
Total Amino acid %		2.20		0.92		0.46		1.82		0.34	5.7

RM: Non-essential amino acid in raw Material, Prod**:** Non-essential amino acids in the recipe, **ND:** Not determined.

3.7.5 Mineral composition

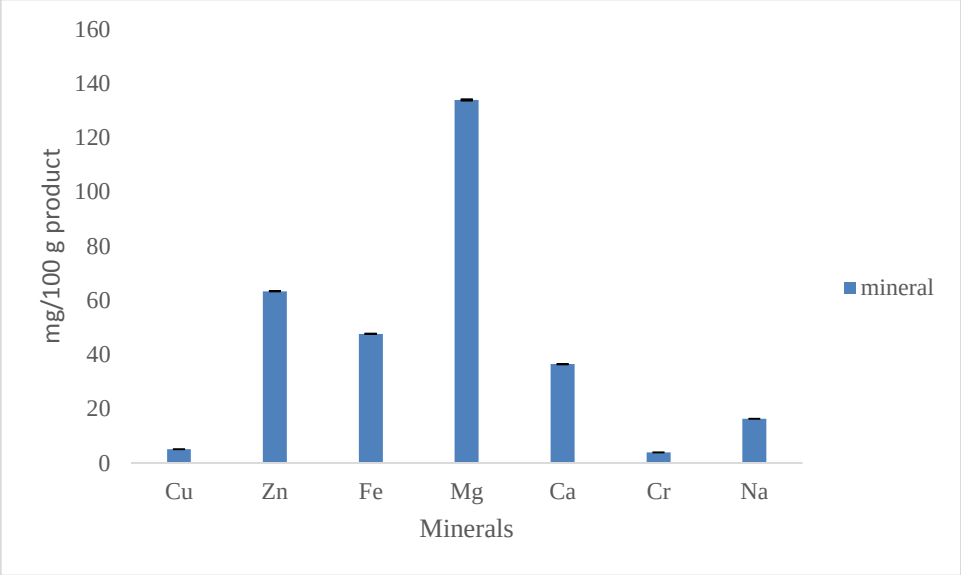

Figure 3.14. Mineral content of the black seed mix (mg/100g)

The mineral composition as analysed by atomic absorption spectrometry (Figure 3.14) shows that magnesium and zinc are the predominant minerals in the black seed mix, followed by iron, calcium, sodium, copper, and chromium.

3.8 Nutritional assessment

3.8.1 Nutritional composition of the black seed mix compared to recommended daily intake for different age groups

Table 3.9 indicates that, if approximately 200 g of the black seed mix would provide the RDI for protein for children of 5-10 of age and approximately 50% of RDI for older age groups. 200g of black seed mix would also provide approximate 50% of carbohydrates and fat for all ages' groups, sufficient fibre for all age groups and approximately half of the daily requirement of calories required for women and children.

Table 3.9. Recommended daily nutritional intake for each age group compared to the composition of the black seed mix (Food and Drink Federation, 2015)

Nutritional fraction	Women (grams)	Men (grams)	Children 5-10 years (grams)	Content in mix (grams/200 g)
Protein	45	55	24	26
Carbohydrate	230	300	220	110
Sugars	90	120	85	
Fat	70	95	70	48
Fibre	24	24	15	34.6
Salt	6	6	4	2.6
Calories	2,000 kcal	2,500 kcal	1,800 kcal	913 kcal

3.8.2 Amino acids

Below, we compare the amino acid composition of the black seed mix to recommended daily intake for adults and children.

3.8.2.1 Amino acid requirements of adults compared with the black seed mix contents

Table 3.10 indicates that the consumption of 200 g of the black seed mix per day by adults could meet the requirements for histidine, isoleucine, and the essential amino acids methionine, cysteine and valine. This intake would also provide approximate 50% requirements for lysine, leucine, phenylalanine or tyrosine.

Table 3.10. Comparison of daily amino acid requirements of adult and amino acid content in the black seed mix (World Health Organisation, 2007)

Amino acid	Estimated mg/70 kg adult per day	Black seed mix (mg/200 g)
Histidine	70	380
Isoleucine	140	800
Leucine	2,730	1700
Lysine	2,100	1080
Methionine	70	380
Cysteine	280	340
Phenylalanine and tyrosine	1,750	1100
Threonine	1,050	2600
Tryptophan	280	Not measured
Valine	26	920

3.8.2.2 The amino acid requirements of children and adolescents compared with the black seed mix contents

Table 3.11 shows that the consumption of 200 g of the black seed mix per day by children at ages of 3-14 would meet the required daily intake of all amino acids except tryptophan which was not measured. A smaller portion of 100g would be sufficient for children between1 and 3 years old.

Table 3.11. Daily amino acid requirements of children and adolescents (both boys and girls) compared to the black seed mix contents (mg per average body weight in the table). (Adapted from World Health Organisation, 2007)

Age	Average Weight kg	His	Ile	Leu	Lys	Saa	Thr	Aa	Trp	Val
1-2	10	150	270	540	450	220	230	400	64	360
3-10	20	240	460	880	700	360	360	600	96	580
11-14	30	360	660	1320	1050	510	540	900	144	870
15-18	50	550	1050	2100	1650	800	850	1400	4800	1400
Black seed mix (mg/200 g)		380	800	1700	1080	720	2600	1100	ND	920

His, histidine; **Ile**, isoleucine; **Leu**, leucine; **Lys**, lysine; **Saa**, sulphur amino acids; **Aa,** aromatic amino acids **Thr**, threonine; **Trp**, tryptophan; **Val**, valine

3.8.3 Fatty acids

The black seed mix also contains a high amount of the nonessential fatty acid oleic acid (5.19g/100g) as well as high amounts of the essential fatty acid, linoleic acid (LA); 100g of black seed mix provides 7.25 g (Table 3.4) of linoleic acid (LA). A 200g portion of black seed mix would provide 14.5 g of LA which would cover the RDI for all age groups. The recommended daily intake (RDI) of linoleic acid (LA) for children boys and girls from 1 to 8 years between 5 and 8g a day and the RDI for higher age groups is between 8 and 13 g a day (Howe *et al* 2006). 200g of black seed mix provides 0.28g of the essential fatty acid linolenic acid (ALA) (Table 3.4) which is less than recommended daily intake for all age groups. The RDI daily recommended intake children from 1 to 18 years is 0.5-0.8 g per day and for adults it is 0.8-1.3g per day.

3.8.4 Minerals

The recommended daily intake (RDI) of magnesium for children between 5 and 16 years is 200- 400 mg/day (Story and Stang, 2005), which a 200 g portion of the Black seed mix would provide (280mg/200g) (Figure 3.15). The RDI for zinc is 8-9 mg/day and 200g of the black seed would provide more than the RDI for zinc (140 mg/day) (Figure. 3.15). The black seed mix contains a high concentration of iron (100mg/200g) (Figure 3.15) which easily surpasses the RDI for adults (17-19 mg/day) and children (13-16 mg/day), (Story and Stang, 2005).

Zinc, magnesium and iron act as cofactors to the antioxidant enzymes in the body such superoxide dismutase, catalase and glutathione peroxidase that counteract the effect of reactive oxygen species that cause damage to cells and DNA *in vivo* (Rowland, 2012). These results show that the black seed mix contain adequate supply of minerals that may assist the antioxidation processes in the body. Adequate levels of iron should address anemia that is prevalent in cases of malnutrition.

3.9 Conclusions

3.9.1 Recipe for the black seed mix

In the current study, the composition and nutritional properties of a seed mix is inspired by an old Egyptian jam called *Mofataka* was developed. Various recipes are used to make *Mofataka*, but the most familiar one is made of a mixture of seeds (peanuts, fenugreek, and sesame), ghee (butterfat), black honey, and wheat flour (Table 3.12). To prepare it, the black honey (treacle) is heated until boiling; then the fenugreek is added and mixed until it is completely integrated. In another pot, the fat is mixed with the flour and heated and stirred until the flour changes colour from white to yellow. Then the sesame seeds and the ground peanuts are added to the oil and flour mixture and stirred until a caramel colour is achieved. Finally, the mixture is added to the treacle and fenugreek, and all is mixed

together and cooled down. This old recipe is used in order to fatten the girls. Fatness was considered as a sign of beauty.

However, in the new recipe, the main purpose was to develop a product with adequate amount of protein. The approach followed was to gain information in published literature of the proximate composition of each of the components of the original recipe. Based on information of basic human nutritional requirements for each age group obtained from literature, a new recipe was formulated to optimise the nutritional content of the Black Seed Mix within the limits of taste and texture. The nutritional content of the recipe was calculated using an algorithm created in excel to calculate the contribution of fat, protein, carbohydrate, water and ash of each ingredient (Table 3.5, 3.7 and 3.8). Some major changes were made to the old recipe to suit our purpose. We replaced the flour with millet and replaced fat with black seeds. These changes in the ingredients were necessary to improve the nutritional value.

Table 3.12. Improved black seed mix recipe compared to the traditional recipe.

Traditional	**%**	**New recipe**	**%**
Butterfat	16	Black seeds	2.9
Fenugreek	4	Fenugreek	8.8
Flour	0.8	Millet	5.8
Peanut	6	Peanut	10.5
Sesame	3	Sesame	10.5
Treacle	70	Treacle	61.4
Total	99.8	Total	99.9

3.9.2 Nutritional composition

The proximate composition of the final product made in practise was analysed by approved AOAC methods and the results were compared to nutrient calculation of the recipe using

published composition of individual ingredients. The amino acid composition was analysed by HPLC, the fatty acid composition by GC and minerals by atomic absorption spectroscopy. To summarise the nutritional value of the black seed mix, a sample of 200 g would provide:

- RDI for protein for children of 5-10 years of age
- RDI for all amino acids for children 5-10 years of age except tryptophan
- 50% of RDI for protein for age groups older than 10 years
- RDI for histidine, isoleucine, methionine, cysteine and valine for all age groups
- 50% RDI for lysine, leucine, phenylalanine or tyrosine for all age groups
- 50% of RDI for fat for all age groups
- 50% RDI for carbohydrates for children and woman
- RDI for linoleic acid for all age groups.
- RDI for fibre for all age groups
- RDI for magnesium, zinc and iron for all age groups
- 50% of RDI for calories for children and women

Although 200g would be a relatively large portion for small children to consume and a more feasible size would be 20-50g per day, the nutritional value of 200g is used in this study as a uniform quantity for all age groups. Nevertheless, even smaller portions would add significantly to daily nutrition.

One of the exceptional nutritional advantages of the black seed mix is the high content of iron. Harris *et al*. (1939) demonstrated that the chemical availability of iron in three grades of treacle as determined by the dipyridyl method was 97, 85 and 54% respectively. The availability as determined by biological procedures (rats) was 90, 80 and 50% respectively. These results confirmed the biological availability of iron in treacle

which indicate its use as an inexpensive nutritional supplement, which would be essential to address the prevalence of anemia in malnourished individuals. Of course this benefit has to be temepered by the potential of the iron to impact on shelf life and the associated sensory changes accompanying the Fe^{2+} to Fe^{3+} transition.

Another point to be addressed is the high sugar content of the black seed mix. A portion size of 200g contains 110g sugar which would provide approximate 50% of the RDI of carbohydrates required for women and children. Although most of the carbohydrates consist of sugars derived from treacle, and intake of sucrose is associated with negative impact on health such as diabetes and obesity, a recent study reported the beneficial effect of treacle on blood glucose, which is attributed to the presence of polyphenols in treacle. Wright *et al*. (2014) reported 5-20% reduction in postprandial glucose responses in human volunteers that consumed treacle compared to untreated controls as assessed by accredited glycaemic index (GI) testing. The authors concluded that the high correlation of treacle to available carbohydrate on glucose response indicated that treacle may have a direct effect on carbohydrate metabolism, despite containing a high concentration of sucrose. This effect was attributed to the high polyphenol content, which had been shown to moderate carbohydrate metabolism *in vitro* by inhibition of glycolytic enzymes (Hanhineva et *al*., 2010). It should be kept in mind that the new product design in the present study was targeted at the malnourished who need a greater sugar intake for energy.

3.9.3 Availability of raw materials

This information provided in section 3.2.7 supports the feasibility of larger scale manufacture of the product for distribution for humanitarian purposes to alleviate malnutrition in poor countries, or alternatively for commercial exploitation in developed countries. However, the optimised recipe would also be valuable for use in small

communities that have their own local supplies of fenugreek and black seeds. This finding has not been reported in the literature. This food product is widely made and consumed in households in Middle Eastern countries, and knowledge and optimisation of its composition could be useful in providing a source of nutrition for malnourished children.

Finally, it is quite reasonable to say the black seed mix could help in the solution of a serious global problem, which is malnutrition. It can be a significant part of a daily healthy diet that provides a decent nutritional balance.

CHAPTER 4

Characterisation, Sensory Evaluation, and Shelf Life of the Black Seed Mix

4.1 Aims

1. Characterisation of the freshly prepared Black Seed Mix product based on water activity, texture and sensory properties.
2. Determination of shelf life based on water activity, texture, and fat oxidation.

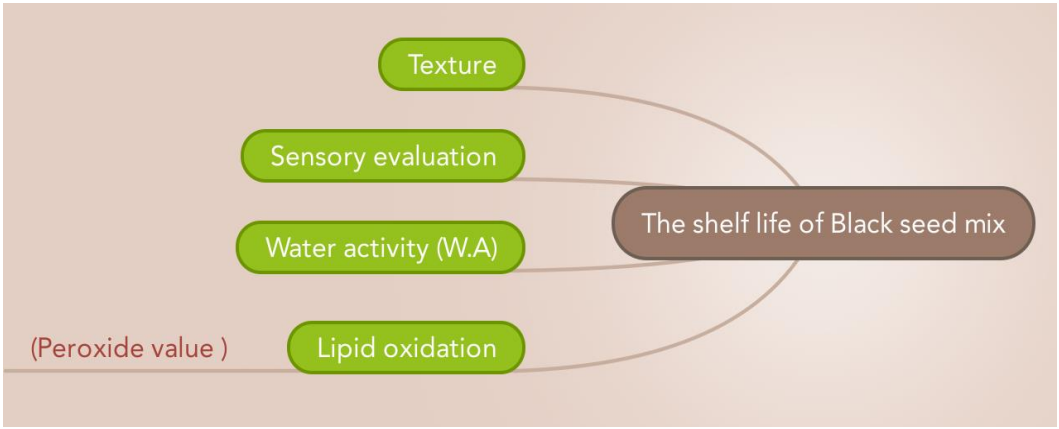

Figure 4.1. Lay out of the chapter

4.2 Introduction

4.2.1 Factors affecting shelf life and measurement methods

4.2.1.1 Water activity (aw)

The principal factor in the determination of food shelf life is its water availability having an effect on food components and safety, together with quality, texture, and the food products' sensory properties with regards to water interaction (Fontana, 2005). Water availability also has effect on Maillard reactions and spontaneous autocatalytic lipid oxidation reactions, together with enzymes and vitamins activities (Sandulachi, 2012).

Sugar-based confections which have lower moisture contents can boast of a longer shelf life. An essential parameter used commonly in characterising microbial stability and texture, as well as the water migration when stored, is the relative vapour pressure or water activity. Water activity (WA) is the partial vapour pressure of water in a food product divided by the standard state vapour pressure of water. (the standard state is the patial

vapour pressure of water at the same temperature as the product). Much research and development has been devoted to reducing water activity in food by drying and freezing, together with adding of sugar or salt to the food product (Fontana, 2005). The quantity of water present in a food product is not what determines the water activity but the manner by which the water is bound. The term is utilised in making reference to the link between moisture content (%) and water activity at a specific temperature. The link between moisture content and water activity assists in gaining a greater understanding of the physical properties and moisture migration, as well as the shelf-life of foods (Fontana, 2005).

The range of water activity is from zero when water is absent to 1.0 when there is presence of pure water. Higher WA substances appear to lend support to the presence of more microorganisms. The requirement for bacteria is at least an WA of 0.91 whilst that of fungi is at least 0.7, thus water activity can be utilised in making prediction on which microorganisms might cause possible spoilage and infection (Roos, 1993). Water activity is used by food designers in the formulation of shelf-stable food. The description of water activities is sometimes described as “bound” and “free” amounts of water present in a product. The measure of the tightness of “bound” water and the relationship to the work needed for water removal from the system is known as water activity (Nelson Court, 1995). The relationship between water activity and moisture content at a given temperature is called the moisture sorption isotherm (Bell and Labuza, 2000). Moisture sorption isotherms are sigmoidal in shape for most foods as shown in Figure 4.2.

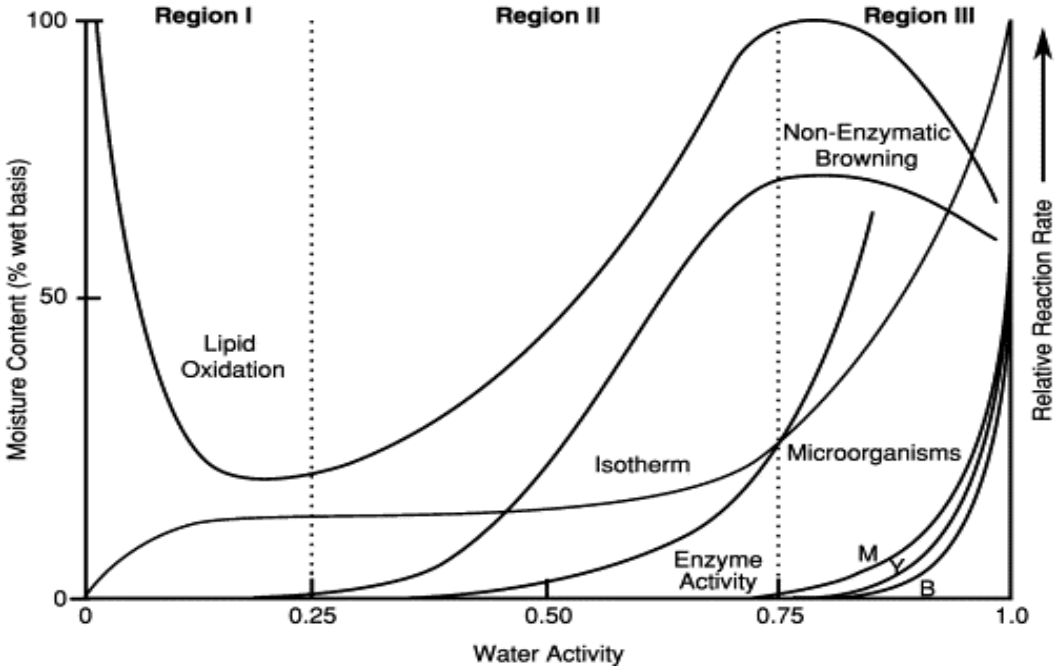

Figure 4.2. Typical moisture sorption isotherms depicting the relationship between water activity and moisture content for most foods (Fontana 2005).

4.2.1.2 Texture analysis

There are certain facts that determine food texture such as physical and physicochemical properties, as well as the complicated features that make up the human senses (Peleg, 1987). Through objective or instrumental and intrinsic subjective or sensory tests, the measurement of texture can be undertaken. Texturometers are among the instrumental test devices. These are capable of duplicating mastication conditions and offer correlations capable of making representative sensory texture evaluations (Szczesniak, 2012). Hardness and cohesiveness, together with viscosity, elasticity, and adhesiveness, as well as brittleness, chewiness, and gumminess are all mechanical attributes relating to measured food and the applied force reactions. Good relationship between instrumental values and subjective assessment can be made by correlating the texturometer data and that derived from a trained texture profile panel.

The force which the molar teeth apply in the compression of food is related to hardness whilst the ability to break down food after the incisors has bitten it is related to

fracturability. A standard rating scale for food texture quantitative evaluation was established by Szczesniak *et al.,* (1963) with regards to hardness and brittleness, as well chewiness and gumminess, viscosity, and adhesiveness. A selected food product based on availability, familiarity, and textural constancy characteristics is represented on every point on the scale. A report on the relationship between sensory and the texture's instrumental (texturometer) evaluations with the use of developed scales was also established (Szczesniak *et al*., 1963). Water activity influences texture since a product that has high water activity usually has soft texture whilst conversely a product that has low water activity usually has hard texture (Hough *et al*., 2001).

4.2.1.3 Sensory evaluation

Sensory evaluation refers to a tool used by food technologist to evaluate the sensory property of food from a consumer perspective and can deal with sight, smell, taste, touch, hearing etc. Sensory description of food is different from chemical analysis of food. The language used in the sensory description is more global, less accurate and uses published definitions of sensory attributes (Sune *et al*., 2002).

4.2.1.4 Lipid autoxidation

Fat and oil play a very important part in the flavour, aroma, texture, and natural properties of foods. The qualities of the oil or fat in the food play an important role in the shelf life of a product. Fat oxidation is the most common chemical change that affects the aroma, flavour, texture, nutritional quality of food products and consumer desirability. Foods with a high amount of poly-unsaturated fatty acids (PUFA) are highly susceptive to food oxidation (Frankel, 2005). Lipid oxidation is affected by temperature, water activity, surface area, and oxygen concentration (Fennema *et al.*, 2007). Lipid oxidation causes flavour changes, often resulting in rancidity, which consist of oxidative and hydrolytic rancidity. Oxidative rancidity is caused when unsaturated fatty acids react with oxygen to

form peroxides. Peroxides form a complex mixture of ketones, aldehydes, odours, acids, and alcohols that cause a rancid or off-flavour of the fat or oil (Okaka and Okaka, 2001). Hydrolytic rancidity occurs as a result of a reaction of the lipid resulting in hydrolysis of the triglycerides and free fatty acids, which can also give a disagreeable flavour (Sebranek and Neel, 2008).

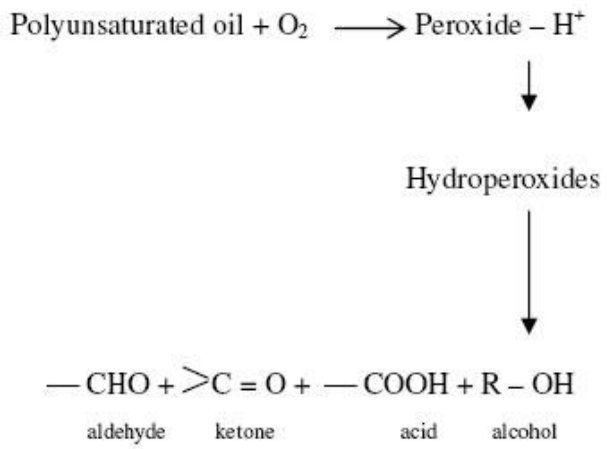

Figure 4.3. Oxidative rancidity (Okaka and Okaka, 2001)

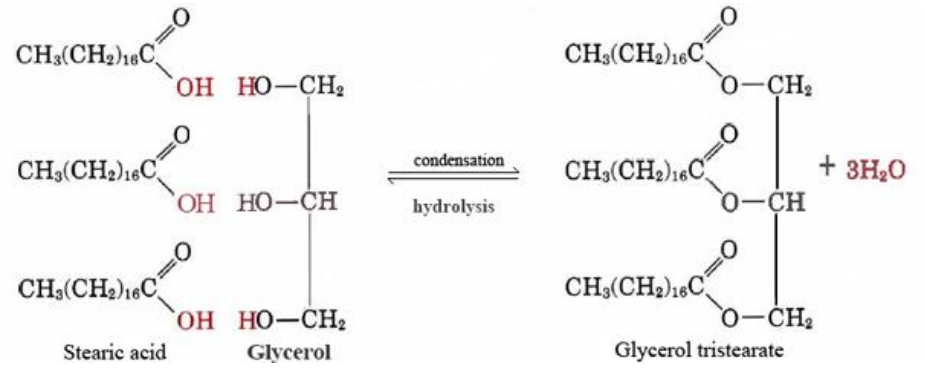

Figure 4.4. Hydrolytic rancidity (Sebranek and Neel, 2008)

Lipid oxidation or auto-oxidation causes changes in the nutritional, sensory, and chemical properties of a product. The free radical chain reaction of lipid oxidation can be described in three steps: initiation, propagation, and termination (Sahoo, 2013).

Initiation: $RH \longrightarrow R^{\bullet} + H^{\cdot}$

Propagation: $R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$

Termination: $R^{\bullet} + R^{\bullet} \longrightarrow RR$

$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR$

$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2$ (non-radical products)

RH refers to a lipid, R to a lipid radical, and ROO to a peroxyl radical.

4.2.1.5 Peroxide value (PV)

The peroxide value (PV) test is one of the most common tests used to measure the peroxides and hydroperoxides present in the initial stage of lipid oxidation in oil containing food products (Fennema *et al.*, 2007). The peroxide milli-equivalents present in 1 g of oil are measured by titration with iodide ion. The peroxide value of purified oil is 0.05 meq/ kg; the maximum peroxide value is 1 meq/kg (Sahoo, 2013).

4.3.1.6 Accelerated shelf life tests

Accelerated shelf-life procedures of confectionary are often attempted to shorten the duration of product development. This is often conducted at higher temperature (37°C) than ambient with the assumption that chemical reactions that cause product deterioration will be accelerated. However, accelerated shelf life results do not always mimics that of ambient conditions due to the following reasons as described by Labuza and Schmidl (1985):

• Phase changes from the melting of fats, and change in solvent properties.

• Phase changes from the melting of fats, and change in solvent properties.

• Crystallisation of amorphous carbohydrates.

• Increased water activity.

Subramaniam and Kilcast (2000) advise that if time pressures do not allow testing of shelf life at ambient storage conditions, then comparisons can sometimes be made between the test product and an equivalent product of similar structure for which a shelf-life has previously been established. In the present study we will compare the textural properties to that of Halwa during accelerated and ambient shelf life studies (Itagi *et al*., 2013). Halwa is an Indian sweet confection made with various kinds of fruits, vegetables, grains, nuts, lentils, wheat, mung beans and chick peas, with a proximate composition similar to that of the black seed mix.

4.3 Materials and methods

The materials and methods used within this study were described in Chapter 2 (sections 2.2.3)

4.4 Results

4.4.1 Water activity (a_w)

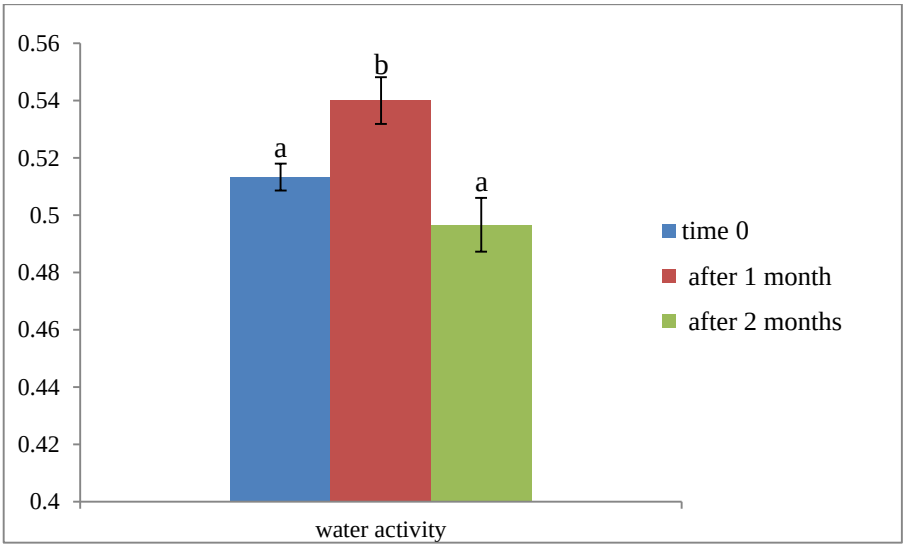

Figure 4.5. Water activity of the black seed mix during storage Blue A fresh sample, red: after one month and green after two months of storage. Bars are means of three measurements and error bars are standard deviation whilst different letters (a, b, **c** indicate significant differences ($p< 0.05$)

The results of water activity enabled us to categorise the black seed mix product as a chewy, sweet candy product based on its water activity of 0.52-0.55 (Figure 4.5), low moisture content of 8.5%, and high sugar content (55.4%) (Chapter 3, Figure 3.14), which is based on the categorisation by Fontana (2005) as depicted in Table 4.1.

Table 4.1. Water activity of confectionery products (Fontana, 2005)

Products	Water Activity	Moisture	Total Sugars
Boiled sweets	0.25-0.40	2-5%	35-60%
Caramels Toffees Fudge	0.45-0.60	6-10%	40-70%
Chewy sweets	0.46-0.60	6-10%	40-60%
Nougat	0.40-0.65	5-10%	30-60%
Marshmallow	0.60-0.75	12-20%	40-65%
Gums Jellies Liquorices	0.50-0.75	8-22%	30-75%
Candied fruit	0.70-0.80	20-30%	35-100 %
Jams	0.80-0.85	30-40%	0-70%

The water activity of the black seed mix that was measured freshly and after 2 months' storage e show a significant increase from 0.45 to 0.5 after one month and a decrease to the initial value after two months of storage at 37^{o}C (Figure 4.5). However, the increased value still falls within the range for chewy sweets (0.46 – 0.60) as shown in Table 4.1 and the increase is still within acceptable limits for chewy sweets such as fudge and caramel (Nelson, 1995).

The increase in water activity could be caused by temporal water migration in the product with associated changes in water binding. Ergun *et al.* (2010) describe the following: "a difference in water activity, either between candy and air or between two domains within the candy, is the driving force for moisture migration in confections. The black seed mix product would be stable with respect to microbial growth according to the

diagram described by Fontana (2005), (Figure 4.2 in the introduction), which shows that microbial growth is very low at a water activity level of 0.5-0.6.

4.4.2 Texture analysis

Table 4.2. Changes in texture of the black seed mix during storage

Time	Fracturability N	Cohesion Strength N	Hardness N
0 time	2.47±0.09[a]	0.55±0.00[a]	1.6±0.08[a]
1 month	8.54±0.07[b]	4.92±0.02[b]	73±0.20[b]
2 months	5.47±1.14[c]	4.07±0.12[c]	16±0.14[c]

Values are means ± standard deviation. Means in the same column not followed by the same superscript are significantly different ($p< 0.05$). 0 time: A fresh sample, 1 month: one month of storage, 2 months of storage

After one month of storage, the hardness, fracturability and cohesion strength were increased compared with the initial measurements. Although the changes are statistically significant, the values after one month still fall within the range of rating scales for confectionary products established by Szczesniak, 2012,. For example, hardness of 1 N correlates to a rating scale of 3 representing fudge and hardness of 80 N correlates to a rating scale for peanut chew (scale 6). That means the increased hardness measured after 1 month of storage was still acceptable and similar to that of an established product (peanut chew). A fracturability of 2 N was similar to that of peanut brittle (scale 2) whereas increase in fracturability to 8N was similar to a rating scale of a peanut chew (scale 3). Again, the increase in fracturability after one months' storage resulted in the texture of a known confectionary product. No correlation was available for increase in cohesion strength. This interpretation of results using available literature indicates that the changes

in texture of the black seed mix after 1 months' storage still resulted in a product that falls within an acceptable range.

Although the hardness, fracturability and cohesion of texture increased after 1 months' storage, the values decreased again after two months of storage. The results correspond to the values for water activity (Table 4.2) with water activity increasing after 1 month and decreasing after 2 months. The concept of glass transition that is determined by moisture migration and water activity is a critical parameter related to storage stability of confectionary (Nelson, 1995). Confections with low moisture content, such as the black seed mix, may contain sugars in the amorphous or glassy state. Differences in water activity, either between candy and air or between different domains within the candy, is the driving force for moisture migration in confection. Furthermore the different physical states (crystalline or amorphous) of sugars might not be in equilibrium which could lead to phase changes within the product. As long as the confectionary products remain below their glass transition temperature the texture remains uniform but if they absorb moisture from the atmosphere a phase transition state occurs leading to an increase in hardness of texture (Nelson, 1995). This kind of phenomenon might explain the increased hardness in texture of the black seed mix after 1 months' storage at 37 °C. Hartel *et al*, (2011) reported that the phase transition temperature for chewy candies lies between 30 and 40 °C, which supports this explanation.

As sugar crystals form due to phase transition, the moisture is forced back into the atmosphere (Lees, 1965), which could explain the reduced water activity in the product after 2 month's storage. The product texture might have reverted back to an amorphous state which explained its softer texture after 2 month's storage. These changes in texture during storage could be controlled by optimising packaging conditions (Nelson, 1995) and storage below the glass transition temperature (30 °C).

4.4.3 Peroxide value (VP)

Figure 4.6 shows the increased peroxide value during storage time. There is no significant difference between the fresh sample (0 time) and the sample after one month of storage. However, the oxidation values in the sample after two months of storage are significantly increased.

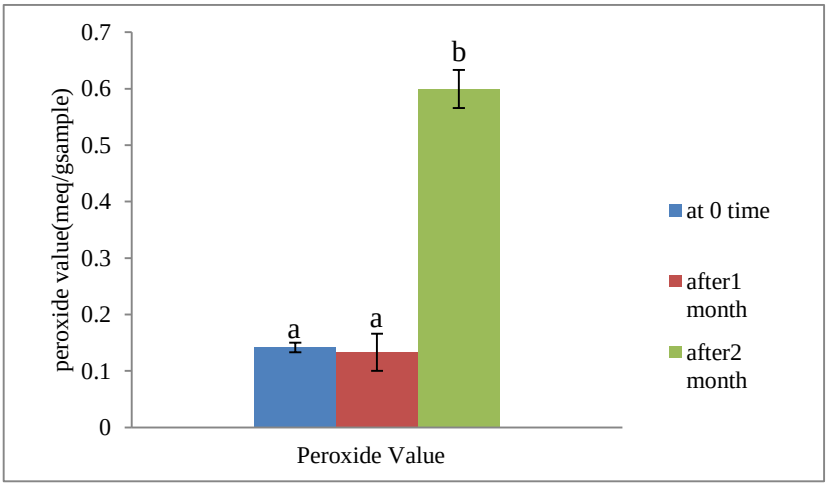

Figure 4.6. Peroxide value during storage at 37°C. 0 time: A fresh sample, 1 month: one month of storage, 2 months of storage. Bars are means of three measurements and error bars are standard deviation whilst different letters indicate significant differences ($p < 0.05$)

The oxidation of fats after 2 month's storage are likely to be due to the high content of unsaturated fatty acids (linoleic acid, Table 3.4) that are susceptible to oxidation possibly as a result of the high mineral content such as iron and calcium (Fig 3.15) that could accelerate the oxidation reactions.

It should be born in mind that the shelf life studies were carried out under accelerated conditions (37°C). The product was stable as regards to POV after 1 month at 37°C, which could equate to 3 months at 25°C for example. The shelf life could be further extended by optimising of packaging. Assuming the results of accelerated shelf life study of the black seed mix is one month it would equate to 2.5 month's storage at ambient temperature. The results were compared to that reported for Halwa (an Indian fruit and

seed confectionary product with similar proximate composition) that had a shelf life of 75 days at 38 °C and 180 days at ambient temperature (Itagi *et al*., 2013).

4.4.4 Sensory evaluation

Figure 2.7 shows the sensory evaluation of the freshly made product given by 11 panellists. The panellists were asked to rate the different attributes on a scale of 1 to 8: from disliked extremely (1) to liked extremely (8). The sensory attributes included the overall acceptability, colour, aroma, texture, bitterness, sweetness, and flavour of the product (Lustre *et al.*, 2007).

The results show that for overall acceptability, a rating of 8 was given by 3 persons, rating of 7 was given by 2 persons, a rating of 6 was given by 3 persons, a rating of 5 was given by 1 person and a rating of 4 was given by 1 person. As for evaluation of the texture, a rating of 8 was given by 3 persons, a rating of 7 by 3 persons, a rating of 6 by 2 persons, a rating of 5 by 2 persons and a rating of 1 by 1 person. Similar patterns were observed for the other attributes. Based on the sensory analysis result, it is reasonable to suggest that the product was overall acceptable by most of the panellists.

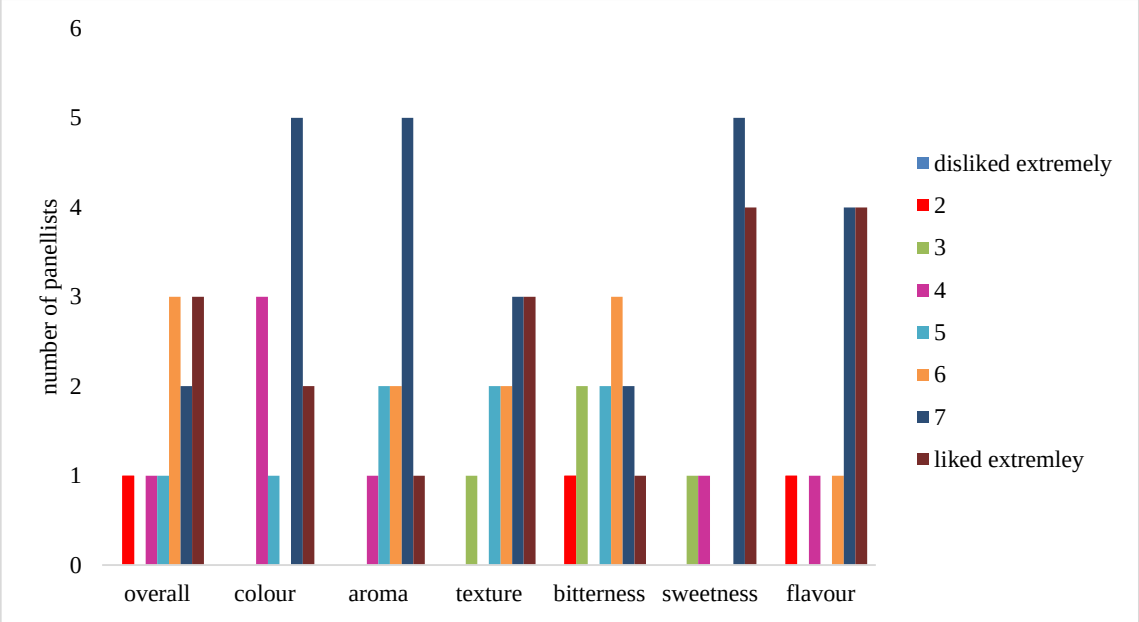

Figure 4.7. Sensory evaluation of attributes of overall acceptability, sweetness, bitterness, aroma, flavour and texture by 11 panellists. The numbers of 1-5 represent the number of panellists that gave a rating on a scale of 1-8 for each attribute. (1 meaning disliked extremely and 8 meaning liked extremely).

4.5 Conclusions

We report here the development of a chewy, sweet confection. Water activity measurements of the fresh product combined with knowledge of sugar content led to categorisation of the product as a chewy, sweet confectionary product. Texture analysis results for the fresh product fell within an acceptable range, and the sensory evaluation proved acceptable by most panellists.

The shelf life of the vacuum-packaged product in accelerated storage conditions (37°C) was acceptable for one month, as determined by the increase in peroxide value of fats after 2 months. This would equate to to 2.5 month's storage at ambient temperature based on published information of a product with similar composition. The hardness and fracturability of the product increased after 1 months' storage, but remained within acceptable limits according to comparisons with data in the literature. The water activity also increased after 1 months' storage but remained within published limits for this

category of confectionary. It is theorised that the sugar in the product underwent a phase transition from amorphous to glassy state at a storage temperature of 37°C, which falls within the published range of glass transition temperature for this category of product. We conclude that shelf life studies have to be repeated at temperature below 29 °C. Furthermore, the textural changes during storage could be improved by an improvement in packaging (Ergun *et al*., 2010).

CHAPTER 5

Effect of Different Thermal Treatments of the Black Seed Mix on Protein Solubility, Maillard Reaction Products and Antioxidative Properties

5.1 Introduction

5.1.1 Lay out of the chapter

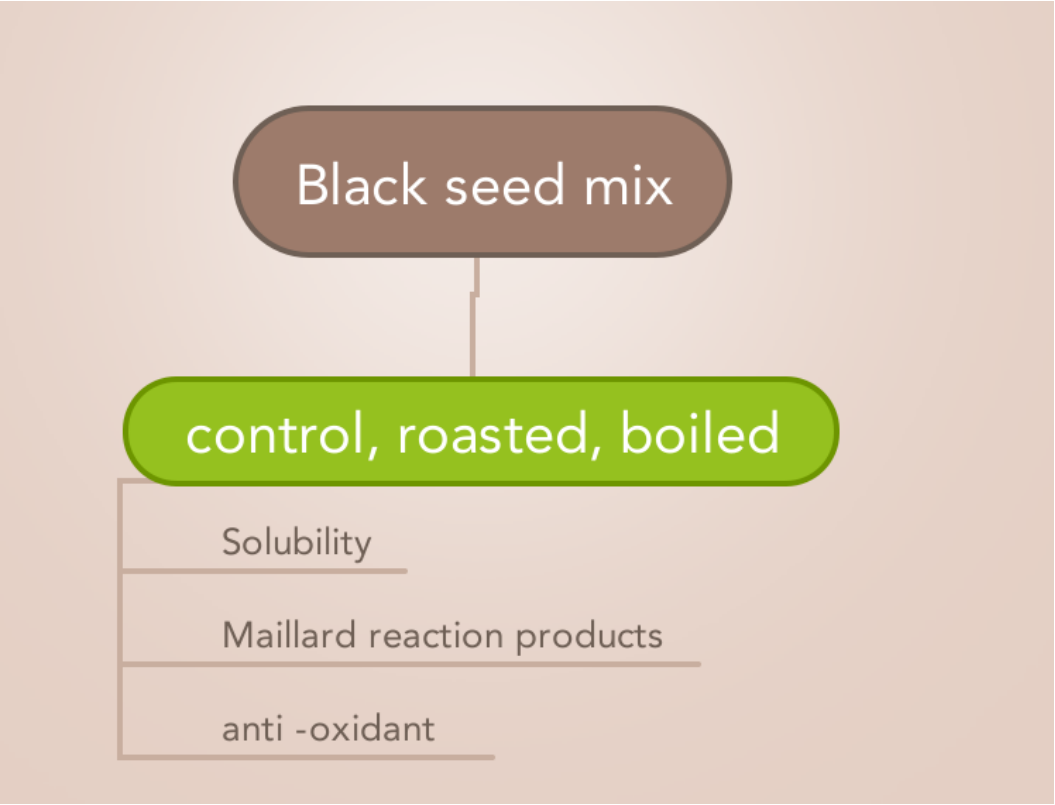

Figure 5.1. Lay out of the chapter.

5.1.2 Aims of the study

This chapter addresses the determination of the effect of different thermal processing methods of the product on Maillard reaction products (MRPs) and antioxidant properties:

- Measurement of the glycation degree and solubility of extracted products.
- Measurement the three different stages of MRPs.
- Measurement of the radical scavenging, ferric reducing and lipid antioxidant properties of different stages of MRPs.

5.1.3 Novelty of the study

Previous studies mainly report the effect of third-stage Mallard reaction products (MRPs) on radical scavenging activity and ferric reducing activity in model systems containing lysine and reducing sugars. The present study will investigate the effect of different thermal processing methods of a nut- and sugar-containing complex food matrix on (MRPs) with respect to radical scavenging, ferric reducing activity and inhibition of lipid peroxidation.

5.2 Overview of Maillard reaction products

The Maillard reaction can be described as a reaction of non-enzymatic glycation which takes place between a protein's amino group and a reducing sugar carbonyl group. The reaction takes place as non-enzymatic browning in food due to procedural outcome like baking or frying, as well as grilling or pasteurisation and the subsequent Maillard reaction products (MRP) that provide cooked foods many of their flavour characteristics (Martins *et al*., 2000). A few decades before, witnessed the study of Maillard reaction chemistry where all the different stages of Maillard reaction took place at the same time and were able to have effect on each other during the reaction process (Wrodnigg and Eder, 2001). The glycation or Maillard reaction process has three stages namely: the early, intermediate, and final stages (shown in Figure 5.2). The glycation reaction leads to cyclisation and dehydration which can be classified into two components and with the first component continuing until the rearrangement of an Amadori takes place while oxidation and dehydration, as well as condensation for the advanced glycation end products development are included in the second component (Nagai *et al.*, 2012).

The important elements present in the regulation of the Maillard reaction are water activity and the type of sugar present, as well as the type of amino acids that are present. Different rates of reactivity are exhibited by different sugars. Hexoses, for example,

manifest less reactivity compared to pentoses. When hexoses are taken into consideration, glucose becomes reactive compared to mannose, which has reactivity that is less than that of galactose (Izydorczyk, 2005). Generally, non-enzymatic browning takes place in majority of foods that have water activities which are in the range of between 0.3 and 0.7. The reaction rate is decreased by increasing water activity as a result of the influence of dilution (Stamp and Labuza, 1983). Again, additional decrease in water activity typically results in an increase in the browning rate, with the exception of the food system where the reduction in sugars and amino acids movement is restricted by it (Eichner and Karel, 1972).

The development of a wide range of reaction products which are of major importance to the foods' nutritional values are made during non-enzymatic glycation. This reaction could lead to the reduction of the nutritional value through decrease in digestibility or through change in the proteins immunoreactivity (Maleki *et al.,* 2000; Chung and Champagne 2001), or possibly through toxic and mutagenic compounds formation. Nonetheless, improvement of protein's nutritional value can be made through antioxidative and antimutagenic products development (Martins *et al*., 2000).

It is possible for Maillard reaction products (MRPs) to potentially change the foods' functional and/or biological properties. The use of MRPs in the form of commercial food additives as emulsifiers in application is the present trend. The interests of a great number of researchers in such disciplines like nutrition and toxicology, together with physiology, and pathology have been attracted for the past twenty years by the Maillard reaction. Glycation effects on protein's structural and functional modification in food, including the physiological and pathological significances of protein glycation present in the biological systems are what have been attracting these interests (Kato *et al*., 1996). The reality of Maillard reaction's positive qualities are found mostly in the food systems. These

qualities may be classified in two categories of sensorial and textural. The desirable colour development and volatile and non-volatile flavour, as well as the aroma compounds present during food preparation are the sensory qualities of the Maillard reaction. Protein solubility improvement and water-holding capacity, together with thermal stability (Kato *et al*., 1996), and emulsifying properties (Shu *et al*., 1996; Kato *et al*., 1996) are all textural qualities of the Maillard reaction. The increasing chain length and content of polysaccharide lead to increase in the conjugated proteins functionality (Shu *et al*., 1996). The development of analytical techniques that have become more sensitive in recent years has led to several studies reporting favourable results of the Maillard reaction present in the food systems like compounds formation with antioxidant (Chuyen, 1998; Wijewickreme and Kitts, 1998) and anti-bacterial (Einarsson and Eriksson,1990), as well as anti-mutagenic and anticarcinogenic properties (Aeschbacher, 1990).

5.3 Chemistry of Maillard reaction

5.3.1 Early stage

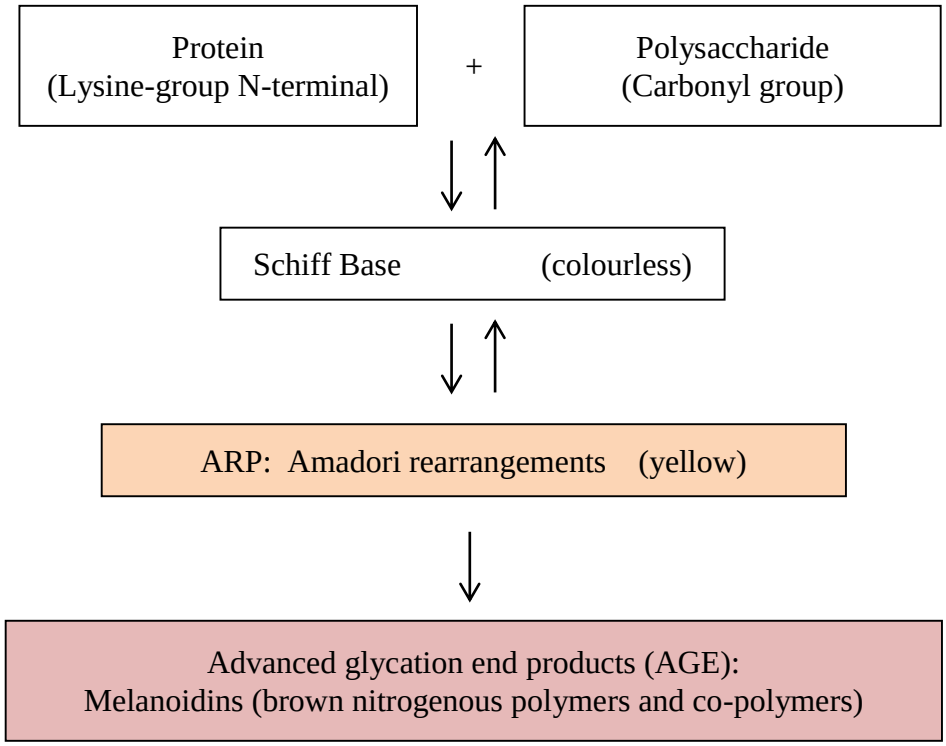

Figure 5.2. Chemistry of the Maillard reaction according to Hodge (1953).

The early stage includes the formation of a Schiff base via reactions between sugar carbonyls and amino acid side chains (particularly lysine and arginine) and the N-terminus of a protein, as shown in Figure 5.3 (Fu *et al*., 1994). The glycation product subsequently rearranges and dehydrates via deoxyinosine, followed by further cyclisation to form Amadori rearrangement products (ARPs). The Schiff base is thermodynamically unstable and is subject to spontaneous rearrangement, forming ARPs as schematically presented in Figure 5.2 (Hodge 1953). The different colour represents the three different stages of the reaction: the blue represents the early stage reaction products; the purple represents the intermediate stage reaction products; and the green represents the final stage reaction products

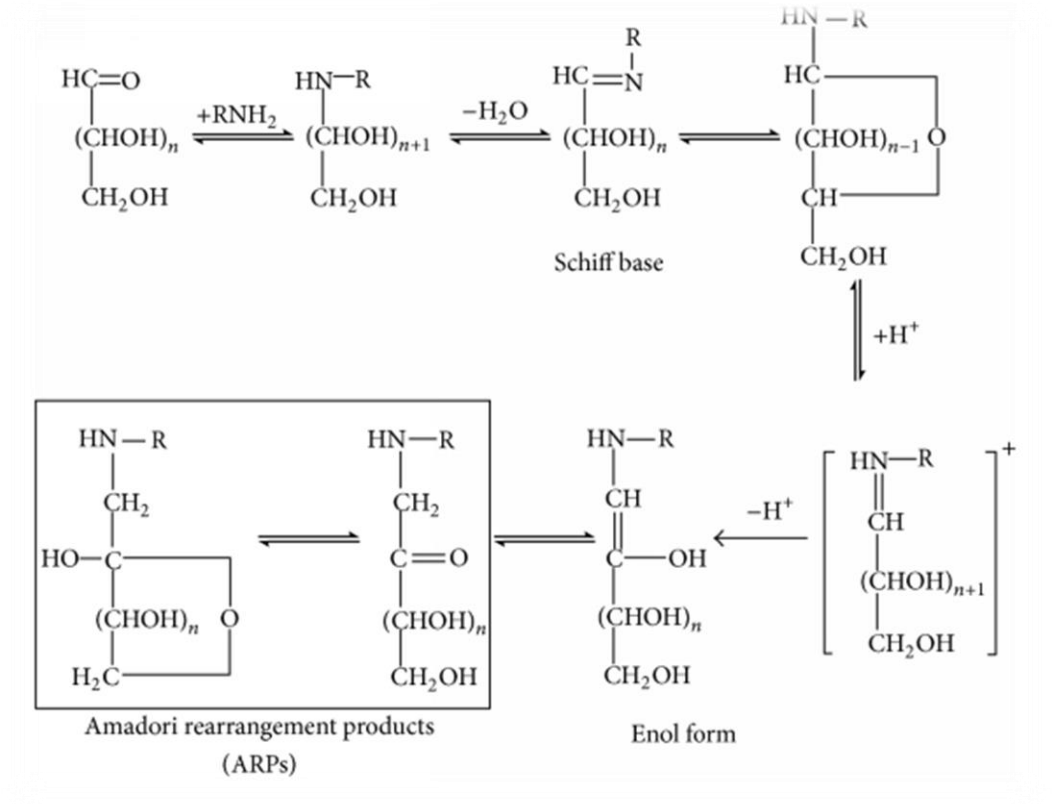

Figure 5.3. Mechanism of Amadori rearrangements in the first stage of the Maillard reaction (Martins et al., 2000).

5.3.2 Intermediate stage

The intermediate stage of the Maillard reaction starts with fragmentation, sugar dehydration, and amino degradation (Figure 5.3). In this stage, the Amadori degradation reaction starts to stabilise during the heating process and can be converted from fragmentation to dicarbonyl compounds. The dicarbonyl compounds provide a majority of colour and flavour in the Maillard reaction (Martins *et al.*, 2000). In this stage, the polymers and heterocyclic compounds are aldehydes formed from Strecker degradation. The Strecker aldehydes can react with Maillard intermediates, and this reaction can contribute to the aroma of food (Ruan, 2009).

5.3.3 Final stage

The advanced stage begins with the deterioration of the ARP by dehydration of the sugar moiety, forming glycation products (Figure 5.2). In the final stage, high molecular weight heterocyclic compounds are formed; they are brown pigments, referred to as melanoidins. The compounds formed are the result of polymerisation for many higher-reactive compounds formed during the last stage in the Maillard reaction (Strelec *et al*., 2008). The benefit of the glycation process is that it improves food quality in terms of both flavour and aroma. In addition, this process increases the amount of antioxidants and antimicrobials in food products. However, the reaction can also cause a loss of nutritional value and the bioavailability of amino acids (Strelec *et al*., 2008).

5.4 Antioxidants

There are three primary types of antioxidants found in nature. These include vitamins, phytochemicals, enzymes and ions such as Fe^{2+} and Cu^{+}. The human body does not produce antioxidant vitamins naturally, so it is essential to include dietary sources of them in our daily intake of food, be it through foods or supplements. Common antioxidant vitamins include vitamins A, C, E, folic acid, and beta-carotene. Phytochemicals are the antioxidants that are naturally used by plants to protect themselves against free radicals caused by UV damage. Studies show that humans who eat sources of phytochemicals also benefit from the antioxidant properties of the plant. Phytochemicals antioxidants can consist of carotenoids flavonoids, allyl sulphides and polyphenols. All whole grains, fruits, and vegetables, contain phytochemicals, whereas processed or refined foods contain a reduced level of free phytochemicals (Lobo *et al*., 2010). The use of natural antioxidants for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, and cancer have appeared during the last 3 decades. A worldwide trend towards the use of natural phytochemicals present in berries, crops, tea, herbs, oilseeds, beans, fruits, and

vegetables has increased. Several herbs and spices have been reported to exhibit antioxidant activity, including rosemary, sage, thyme, nutmeg, turmeric, white pepper, chili pepper, ginger, and several Chinese medicinal plant extracts (Lobo *et al*., 2010; Wang *et al*., 2011).

5.5 Reactive oxygen species

Reactive oxygen species (ROS) can be described as extremely reactive molecules brought about by oxygen's in vivo metabolism. They are developed during aerobic respiration mitochondrial electron transport. A number of them contribute in cell physiology like cell signalling. Nonetheless, some of them can destroy cell membranes and DNA, thereby resulting in membrane lipid peroxidation and reduced membrane fluidity, as well as DNA mutations, and leading to cancer, degenerative, and other illnesses. (Lobo *et al*., 2010). ROS are caused by formation of radicals caused by the reactivity of two unpaired electrons in separate orbits in the outer electron shell of oxygen. The reduction of oxygen through the addition of electrons leads to the formation of a number of ROS including: superoxide; hydrogen peroxide; hydroxyl radical; hydroxyl ion; and nitric oxide (Held, 2014) as presented in Fig 5.4.

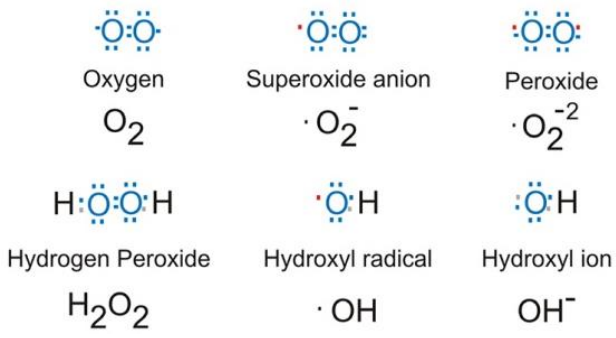

Figure 5.4. Electron structures of common reactive oxygen species. Each structure is provided with its name and chemical formula. The red • designates an unpaired electron (Held, 2014).

One of the causes of hydrogen peroxide formed by ROS is lipid peroxidation, which is one of the most widely used indicators of free radical formation and cause damage to cell membranes *in vivo* as well as in fat containing foods resulting in rancidity and reduced shelf life. Reactions occur as a chain reaction where a free radical will capture a hydrogen moiety from an unsaturated carbon to form water. This leaves an unpaired electron on the fatty acid that is then capable of capturing oxygen, forming a peroxy radical (Figure 5.4). Lipid peroxides are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds (Mensah *et al*, 2014).

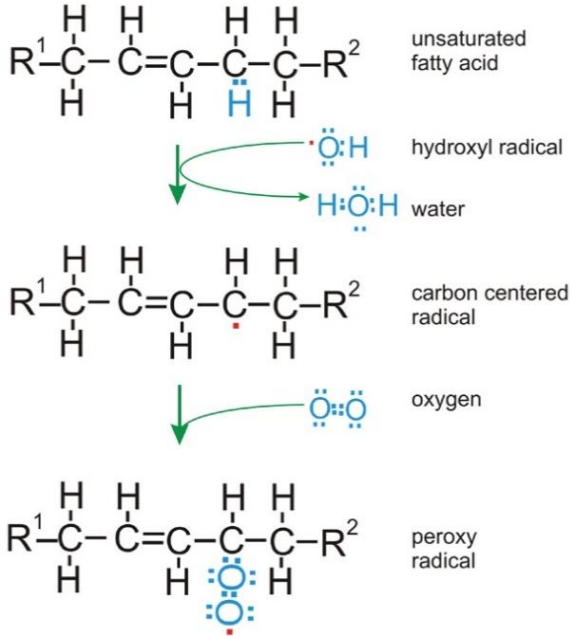

Figure 5.5. Illustration of lipid peroxidation (Held, 2014).

Mammals can counteract the effect of ROS *in vivo* by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), which destroy toxic peroxides. Furthermore, non-enzymatic molecules such as thioredoxin and glutathione play important roles in antioxidant defense systems (Lobo *et al*., 2010). In order for antioxidant enzymes to provide optimum antioxidant activity, they require co-factors such as iron, copper, selenium, magnesium, and zinc (Lobo *et al*., 2010). Figure

5.6 gives a schematic presentation of the interrelationship between some antioxidants and reactive oxygen species (Bahorun *et al*., 2006).

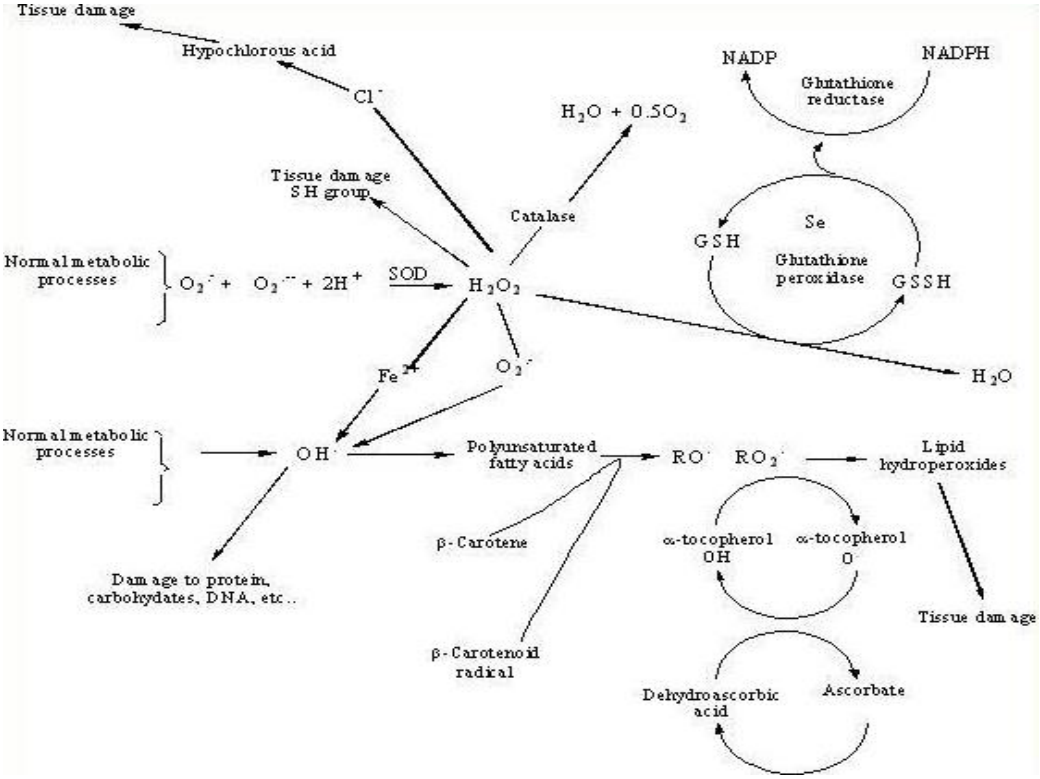

Figure 5.6. Inter-relationship between reactive oxygen species and antioxidants **(**Bahorun *et al*., 2006).

Heat treatment of food containing fat may cause lipid oxidation even when the fat content is comparatively low, resulting in the loss of sensory and nutritional quality and a short shelf life of the food product. Antioxidants are used to retard or slow down the oil oxidation in the food product to improve food quality and to extend the shelf life (Sahoo, 2013). Synthetic antioxidants such as butylate hydroxyanalsoe (BHA), teri-butylhydroquinon (TBHQ), butylated hydroxytoluene (BHT), and propylgallate (PG) are currently added to foods, but evidence has been reported for carcinogenic side effects (Singh *et al.* 2002). Nowadays, increased efforts are directed to find natural antioxidants to prolong shelf life of food, such as plums, grapes, almond skins and Maillard reaction products (Vasavada and Cornforth, 2006).

5.5.1 Antioxidant properties of Maillard reaction products (MRPs)

High antioxidant capacities have been reported for food products that have undergone the Maillard reaction such as roasted flours (Rufian-Henares *et al*., 2009), roasted tea leaves (Zainol *et al*., 2003), beer (Tafulo *et al*., 2010) and roasted coffee beans (Cho *et al.*, 2014). On the other hand, it is reported that naturally occurring antioxidants could be significantly lost as a consequence of thermal processing and storage (Anese *et al*., 1999; Cho *et al*., 2014).

Numerous studies are published on antioxidant properties of MRP's generated in thermally treated model amino acid and reducing sugar systems (Wang *et al*., 2011, Chawla *et al*., 2009, Maillard *et al*.,2007,Amarowicz, 2009, Gu *et al*., 2010). Several mechanisms for the antioxidant activity of MRPs have been identified, including radical chain-breaking activity, metal chelation, reducing powder, decomposition of hydrogen peroxide and scavenging of reactive oxygen species (Gu *et al*., 2010).

MRPs were also reported to enhance the oxidative stability of food products during storage such as bakery (Lerici and Nicol, 1996) and milk powders (Hansen and Hemphill 1984). Hence MRPs that were shown to have antioxidant activities by *in vitro* tests could counteract the effects of ROS *in vivo* and improve shelf life of food products (Fig 5.7).

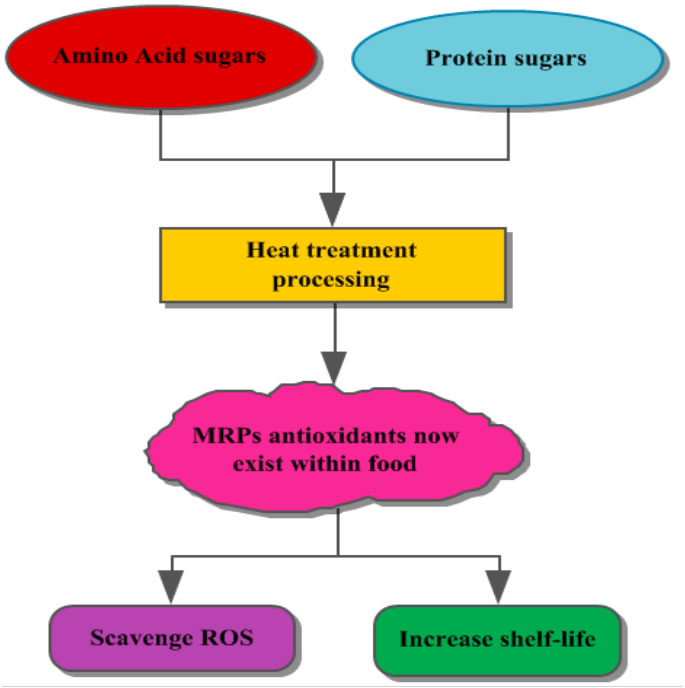

Figure 5.7. MRP antioxidant effects in vivo and in food.

There are conflicting reports in the literature on the correlation between degree of browning of MRPs and antioxidant activity (Amarowicz, 2009, Gu *et al*., 2010 and Vhangani andVan Wyk 2013) and it was demonstrated in model lysine and reducing sugar systems that the antioxidant capacity of MRPs increased in correlation with increasing heating intensity and corresponding increase in browning intensity as measured by absorbance at 420nm. On the other hand, Morales and Jimenez-Perez (2001) reported that the degree of browning as measured by absorbance at 420 nm of a model lysine and reducing sugar system was not directly related to the antioxidant properties of MRPs, however the fluorescence of MRPs correlated well with antioxidant properties. Both research groups used the same methods to measure antioxidant properties. Cho *et al*., (2014) reported significant loss in antioxidant activity of darkly roasted coffee beans compared to lightly roasted coffee beans. The authors reported that slight roasting results in the formation of MRPs and increased release of bound polyphenols from plant cells,

whereas further roasting significantly decreased the total phenolic content and reduction in antioxidant activity.

The aim of the studies reported in this chapter is to investigate the effect of different stages of the Maillard reaction, generated by different cooking procedures in the black seed mix, to the anti- oxidant properties. Only one earlier study correlated fluorescence of intermediate stage MRPs with antioxidant properties (Morales and Jimenez-Perez, 2001), but studies were conducted using a model lysine and reducing sugar system. The novelty of the present study is to investigate this relation in a complex food system (the black seed mix). All previous studies on complex food systems only reported the effect of the browning reaction of MRPs as measured visually or by absorbance at 420nm on antioxidant properties. The present study will also investigate the fluorescence of intermediate stage MRPs on antioxidant activity of the black seed mix prepared by different heating methods.

5.5.2 Overview of methods used to measure antioxidant activity

Different methods are used to measure the anti-oxidative activity of compounds and are classified according to their inactivation mechanism of free radicals as direct and indirect methods. Indirect methods measure the ability of a molecule to reduce a stable, artificial free radical by means of hydrogen donation or electron transfer (Vangani and Van Wyk 2013). Direct methods utilize oxidizable substrates such as lipids or DNA to determine the inhibitory potential of an antioxidant by subjecting these substrates to natural or accelerated oxidation conditions. Direct methods are often used to measure the antioxidant capacity of components in oil containing foods with the aim to prolong shelf life (Zainol *et al*, 2003).

Some researchers advise the implementation of several assays to evaluate the anti-oxidative capacity of food components (Erkan *et al.* 2008, Rzaeizadedeh *et al.* 2011).

However recent publications reported selection of one or two assays as sufficient depending on the purpose (Sergey *et al*., 2005, Thaipong *et al*., 2006 and Zainol *et al*., 2003 and Vangani and Van Wyk 2013).

5.5.2.1 Indirect methods

Several indirect assays are described in the literature to measure antioxidant capacities in fruits, vegetables and cereals including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Leong and Shui, 2002), diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams *et al*., 1995), ferric reducing antioxidant power (FRAP) (Guo *et al*., 2003) and the oxygen radical absorption capacity (ORAC) (Ou *et al.*,2001). These techniques have shown different results among crop species and across laboratories (Thaipong *et al*., 2006). Variations in the extent of different types of antioxidant activity in different plant extracts were also reported by Frankel (2005) some extracts exhibited high radical scavenging but low inhibition of lipid peroxidation. The authors postulated that the differences may be due to different antioxidant mechanisms. Ou *et al*., (2002) reported no correlation of antioxidant activity between the FRAP and ORAC techniques among most of 927 different freeze-dried vegetable samples, whereas these methods revealed high correlation in blueberry fruit (Connor *et al*., 2002). Similarly, Awika *et al*. (2003) observed a high correlation between ABTS, DPPH, and ORAC amongst sorghum products. Taipong *et al.*, (2006) tested ABTS, DPPH, FRAP, and ORAC assays for estimating reproducibility of anti- oxidant activity assays in guava fruits and reported that the DPPH and FRAP assays showed no differences among determinations, while the ABTS and ORAC assays differed among runs. The authors concluded that the FRAP technique showed high reproducibility, was simple, rapidly performed and showed the highest correlation with both ascorbic acid and total phenolics in guava fruits. Sergey *et al.*, (2005) measured the antioxidant capacity of Rubus fruit juice by the DPPH and FRAP assays and demonstrated significant

correlation with reduction of lipid peroxidation *in vivo* in *Drosophila melanogaster* flies. A significant correlation between the phenolic content in fruit juice and both *in vivo* and *in vitro* antioxidant activity was demonstrated.

5.5.2.2 Direct methods

Direct anti-oxidant methods are often used to measure the inhibition of lipid peroxidation in foods. The oxidative deterioration of lipid-containing food is responsible for the rancid odours and flavours during processing and storage, consequently decreasing the nutritional quality and safety of foods. Zainol *et al*. (2003) used the ferric thiocyanate (FTC) method and thiobarbituric acid (TBA) methods to determine the antioxidant power of a Malaysian herb to inhibit lipid peroxidation. The authors reported a positive correlation of the measurements with the phenolic content of the herb. The antioxidative activities were reported to be comparable to that of α-tocopherol (natural antioxidant) and butylated hydroxytoulene or BHT (synthetic antioxidant). Aqil *et al.,* (2006) also correlated antioxidant activity of extracts from different plants to their phenolic content, using the FTC and TBA methods.

5.5.2.3 Methods to determine antioxidant potential of Maillard reaction compounds

Vanghani and Van Wyk (2013) used the FRAP and DPPH methods to determine the relation between heating intensity of fructose–lysine and ribose–lysine model systems and their antioxidant activity. Morales and Jimenez-Perez (2001) only used the DPPH method to correlate the browning intensity and fluorescence of MRPs of model systems of reducing sugars and lysine. Cho *et al*., (2014) used the FRAP and DPPH methods to measure the effect of roasting intensity of coffee beans on antioxidant activity. Zainol (2003) used the FTC and TBA methods to determine the effect of roasting of tea leaves on lipid peroxidation.

5.5.2.4 Choice of methods for determination of antioxidant activity of Black Seed Mix

This study used both direct and indirect methods to determine the effect of different heating procedures on antioxidant activity of the black seed mix. For the indirect method, DPPH and FRAP will be used. Whereas these methods are usually carried out to indirectly assess the nutritive quality of anti- oxidants, the FITC and TBA anti -oxidant methods are often carried out to determine anti oxidative effect related to storage stability of fat containing foods. To measure the antioxidant effect as regards to storage stability of the black seed mix, we decided to use the FITC and TBA methods.

i. DPPH radical scavenging activity

This method measures the ability of antioxidants to reduce a stable, artificial free radical (DPPH) by means of hydrogen donation (Elmastas *et al.,* 2007). The chromogen-radical-containing compound diphenyl-picrylhdrazine (DPPH) has high sensitivity and can directly react with antioxidants from a large variety of samples (Awika *et al.*, 2003). When the antioxidants react with DPPH, hydrogen is donated to form the stable DPPH-H molecule, turning from purple to yellow and the absorbance is measured at 517 nm (Awika *et al.*, 2003).

ii. FRAP (Ferric reducing activity)

In this assay, the presence of an antioxidant in a sample reduces the ferric chloride/ferricyanide complex to its ferrous form. The ferrous form is monitored spectrophotometrically by measuring the formation of a Perl's Prussian blue colour at 700 nm. This assay particularly measures the antioxidative activity of MRPs since the hydroxyl groups of MRPs play a role in the reducing activity through their redox potential of transferring electrons (Vanghani and van Wyk 2013)

iii. FTC (ferric thiocyanate) and TBA (thiobarbituric acid) methods

FTC is a method that measures that amount of peroxide at the beginning of lipid peroxidation, whereby, peroxide is reacted with ferrous chloride to form ferric ions. The developed ferric ions then combine with thiocyanate ions to form ferric thiocyanate. The final compound is red in colour, and the denser the colour, the higher the absorbance. The substance is red and has absorbance at 500 nm. The antioxidative activity of black seed mix will be measured as the ability to donate hydrogen atoms to free radicals and inhibit lipid oxidation. As oxidation proceeds, peroxides are gradually decomposed into lower molecular compounds and measured with TBA reagent. The absorbance of the red reaction product is measured at 532 nm (Zainol *et al.,* 2003). The antioxidative ability of BHT (synthetic antioxidant) will be used as positive control.

5.6 Methods for the extraction of proteins from the black seed mix

A variety of methods exist to extract globulins from plant seeds, such as the use of solutions of 0.5 M sodium chloride, ammonium bicarbonate, iso-electric precipitations, and hot water (Sun and Hall, 1975), the extraction of globulins from Amarinth seed by a combination of 0.5 M salt extract and deionised warm water extract (Romero-Zepeda and Paredes-Lopez, 1995;Marsh *et al.* 2008), the extraction of globulins by using 20 mM Tris, pH 7.2, and further fractionation of the various globulins by different centrifugation speeds 2,000 x g for 11S and 35,000 x g for 7S (Marsh *et al.* 2008).

The purpose of the extraction of proteins from black seed mix in this study was two-fold: (A) to determine the effect of different heat treatments on solubility and Maillard reaction products and B) to determine the effect of different heat treatments and addition of reducing or oxidising agents on immune reactivity of proteins as measured by ELISA (enzyme linked immune sorbent assay) and Western blotting techniques. We decided to

follow the protein extraction method according to the method of Rizzello *et al.* (2009), which includes extraction with hot water followed by centrifugation at 3000 x g for the following reasons:

- The sodium content of the black seed mix is 0.5 mg/litre (Chapter 3 Figure 3.14), and the total ash content is 2.6%, which would provide salt conditions for the extraction of some of the globulins. Using a higher salt concentration for the extraction of globulins (0.5 M NaCl) would affect the binding of proteins to the ELISA plate binding and would require dialysis of high salt solutions (https://www.abdserotec.com/helpful-elisa-hints.html). This could add variability in soluble proteins, which could complicate comparison of the extracts of black seed mix samples prepared by different heat treatments for the purpose of ELISA.
- The extract is centrifuged at a relatively low speed of 1,000 x g, which would allow the 7S and 11 S globulin fractions to remain in the supernatant whilst removing the insoluble material.
- Protein extraction of all black seed mix samples prepared with different heating conditions was carried out under the exact sample conditions. Therefore, concentrations of the respective globulin fractions in each extract will reflect the effect of the thermal processing conditions.

5.7 Materials and methods

The materials and methods used within this study were described in Chapter 2 (2.2.4)

5.8 Results

5.8.1 The stages of Maillard reaction products in peanuts heated with treacle at different heating times

The first and third stages of the Maillard reaction was determined by measurement of absorbance at 305 nm for the first stage and 420 nm for the third stage MRPs.

Figure 5.8 shows the first and third stages of the Maillard reaction as a result of heating treacle mixed with peanuts at 80°C at three different times (2, 3, and 4 minutes). The control was a non-heated sample. Absorbance at 305nm measures the first stage reaction products of the Maillard reaction. The formation of the final stage of the Maillard reaction was measured by absorbance at 420 nm.

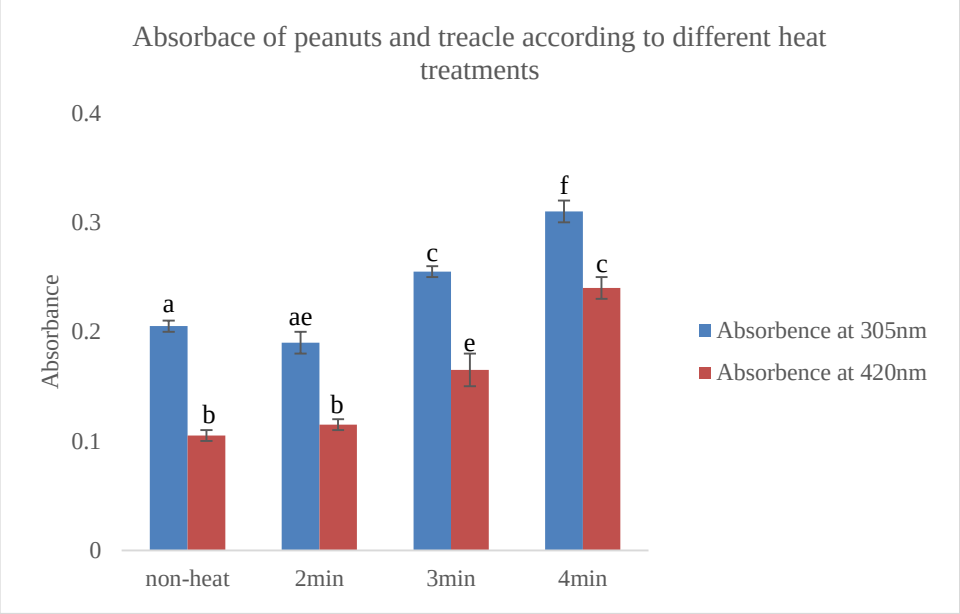

Figure 5.8. Absorbance of the first and last stages of the Maillard reaction in peanut and treacle that was heated for different times (2, 3, and 4 min) at 80°C. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences ($P<0.05$).

Figure 5.8 shows that the non-heated sample and the sample heated for 2 min have a similar absorbance for the first and third stages of the Maillard reaction. However,

samples heated for 3 and 4 min show significantly increased values, demonstrating an increase in both first and third stage MRPs.

The intermediate stage was measured by fluorescence at excitation wavelengths at λ_{EX} = 353 nm and emission wavelength of λ_{EM} = 438 nm. The values were divided by the tryptophan fluorescence of each sample at λ_{EX} = 290 nm and λ_{EM} = 340 nm.

The results are shown in Figure 5.9.

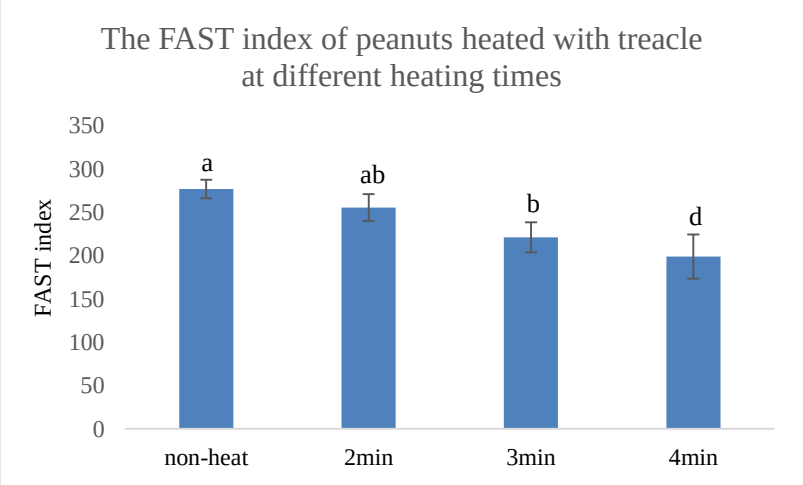

Figure 5.9. FAST index of the fluorescence in peanut and treacle that was heated for different times (2, 3, and 4 min) at 80°C. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences ($P<0.05$).

Figure 5.9 shows high levels of intermediate stage MRPs for non- heated and 2 minutes heat- treated samples. However the values decrease significantly following heat treatment after 3 and 4 minutes. The results indicate that the intermediate stage MRPs decrease as the third stage MRPs increase (Figure 5.8).

5.8.2 Determination of the Maillard reaction: reduction in free amino groups of heat treated peanuts and treacle

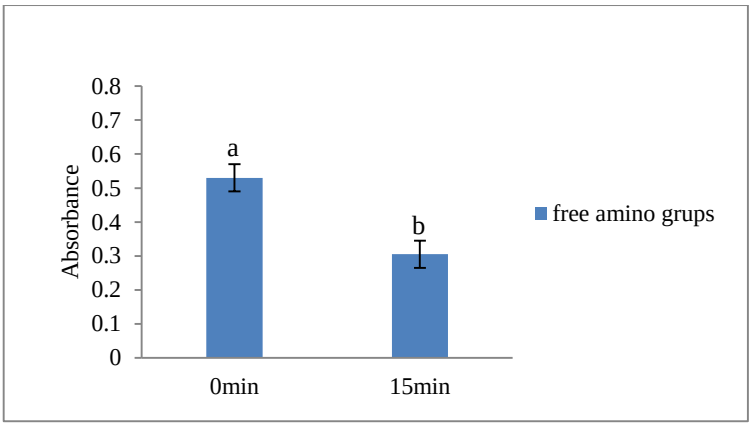

Figure 5.10. Reduction in absorption indicating the reduction in available amino groups of peanut and treacle after heating at 80ºC for 15 minutes. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences ($p < 0.05$).

The glycation of the black seed mix was determined by measuring available free amino groups. As shown in Figure 5.10, the available amino groups of the black seed mix decreased significantly after heat treatment. The Maillard reaction occurred between sugars in the cane sugar treacle and proteins present in the black seed mix. The presence of SDS and mercapto ethanol in the reaction buffer unfolded the protein and exposed the free amino groups to react with the OPA reagent (Chevalier *et al.*, 2001). Figure 5.10 shows that the free amino group decreased from 0.53 to 0.30 after a 15 min heating time

5.8.3 Determination of Maillard reaction products in the black seed mix heat treated by two different methods

Figure 5.11. Appearance of the black seed mix samples under different heating conditions. Sample A: without heat treatment; B: roasted at 150°C for 10 minutes; and C: peanut and sesame seeds boiled and added to the seed mix.

The photographs display a visible change in the colour of the product depending in the heat treatment procedures.

5.8.4 The effects of different heating conditions on the soluble protein in the black seed mix determined using Bradford assay

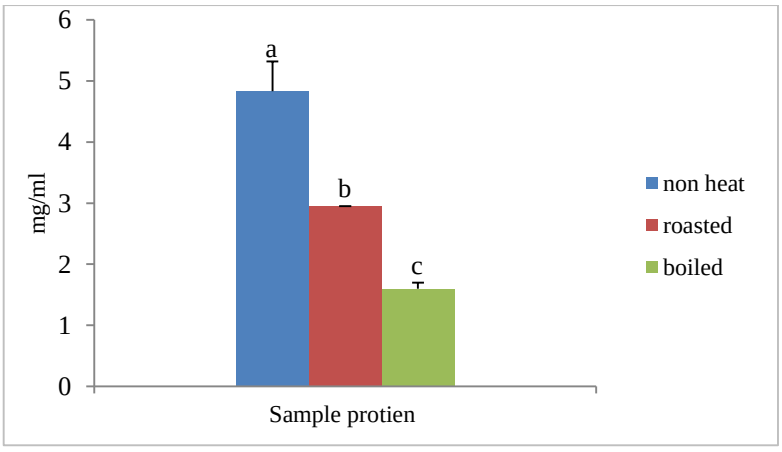

Figure 5.12. Levels of soluble protein in the black seed mix after different heating conditions. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences ($p < 0.05$).

Figure 5.12 demonstrates that there is a significant difference in protein levels between boiling peanuts before heat treatment with the rest of the mix (green) and the other heating conditions: non-heated (blue) and roasted (red).

5.8.5 Maillard reaction products of the black seed mix according to different heat treatments

The protein content of all samples was adjusted to the same level before measurement of absorbance as MRPs will be dependent on the protein content of the samples (see protein determination results in Figure 5.12).

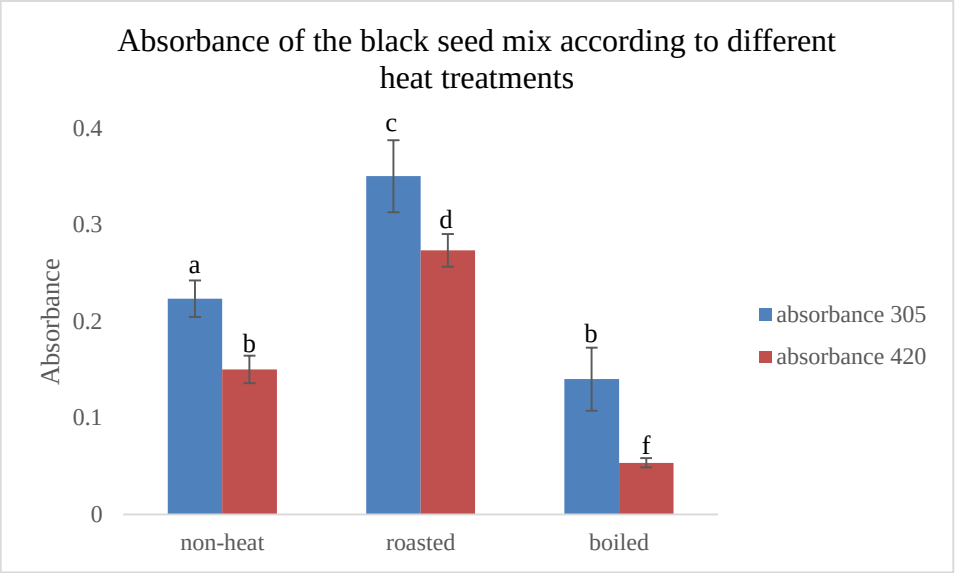

Figure 5.13. Absorbance of the first and final stages of the Maillard reaction in seed mix treated under different heat conditions. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences (P<0.05).

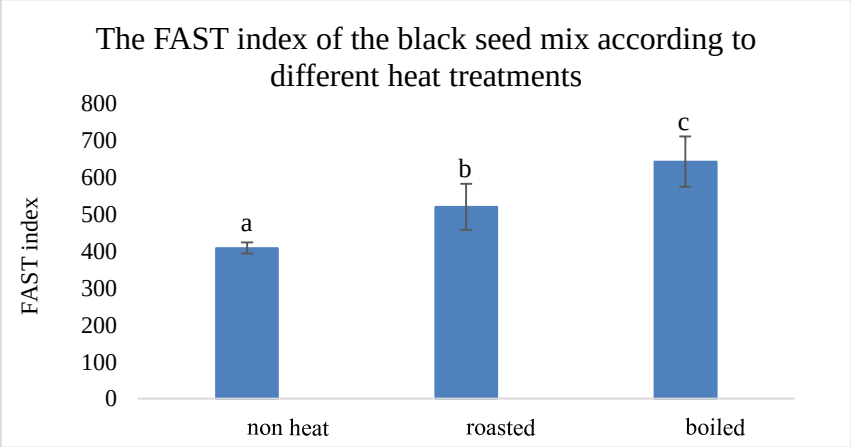

Figure 5.14. The FAST index of the fluorescence of the Maillard reaction in seed mix treated under different heat conditions. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences (P<0.05).

These results in Figure 5.13 indicate an increase in the third-stage MRPs for roasted black seed mix compared to the non-heated control and the boiled sample. However, the absorbance for the boiled sample is lower than that of the control or roasted sample, although the protein concentrations are the same. In contrast, boiled seed mix has significantly higher fluorescence than the roasted sample, indicating intermediate-stage Maillard products. The measurement of fluorescence by the FAST index is calculated relative to the protein content based on tryptophan fluorescence, which eliminates the effect of protein concentration on the results.

5.8.6 Radical scavenging and antioxidant activity of extracts from black seed mix prepared with different heating methods

5.8.6.1 Free radical scavenging assay (DPPH)

Table 5.1 shows the radical scavenging activity as of extracts of black seed mix that had been heat treated by different methods as determined by the DPPH method.

Table 5.1. Free radical scavenging activity of black seed mix processed by different methods expressed as percentage mean ± standard deviation (n = **3**. The roasted sample was heated at 150°C for 10 minutes, the boiled sample means boiling of peanuts before addition to the black seed mix and further heat treatment, Ascorbic acid served as a positive control. Different letters indicate significant differences (P<0.05).

Sample	Radical scavenging activity (%)
Vitamin C	67±0.006a
non- heated sample	$22\pm0.008_{b}$
roasted sample	$54\pm0.030_{c}$
boiled sample	$53\pm0.008_{c}$

The results in Table 5.1 show significantly improved radical scavenging activity for the roasted and boiled sample compared to the non-heated black seed mix sample. The results support the findings of Cho *et al*., (2014) who reported a 15-20% increase in radical scavenging activity of lightly roasted and medium roasted coffee beans compared to the

control. The authors correlated the increase in radical scavenging activity with an increase in phenolic content in lightly roasted coffee bean extracts. However, the authors reported a decrease in radical scavenging activity with darkly roasted coffee beans, associated with decrease in phenolic content.

The results also correspond to findings reported by Vhangani and Van Wyk (2013) of increased radical scavenging activity corresponding to increased heating intensity of a MRP model system. Non heated fructose and lysine heated for 15 minutes at 60°C showed radical scavenging activity of 8%, which was increased to 76% after heat treatment at 121°C for 15 minutes. Gu *et al.*, (2010) reported that the radical scavenging activity of casein -glucose model systems increased by more than 50% following thermal treatment to form MRPs. It should be kept in mind that model systems do not contain phenolic compounds, so the radical scavenging activity is associated with only MRPs and not the phenolic content.

A feasible explanation for the increase in radical scavenging activity observed in roasted and boiled black seed mix, could be due to the release of phenolic compounds, but also due to the increase in MRPs. There would be a fine balance between radical scavenging properties of MRPs and released phenolic compounds in thermally treated black seed mix, which would be affected by the degree of heat treatment.

5.8.6.2 Ferric Reducing Antioxidant Potential Assay (FRAP)

The reducing power assay measures the ability of a substance to reduce ferric ions to ferrous ions. This assay particularly measures the antioxidative activity of MRPs since the hydroxyl groups of hydroxyl groups of MRPs play a role in the reducing activity through their redox potential of transferring electrons to iron (Gu et *al.*, 2010). The absorbency of the samples at 593nm were compared to Iron (II) sulfide standard curve and the FRAP values are expressed as Iron (II) sulfide equivalents which is y=0.086x+0.043

In this test the standard curve (Figure 5.15) revealed a linear relationship between ferrous sulphatesulphate ($FeSO^4$) standards in the range of 0.8 - 10 mM and absorbance at 593 nm

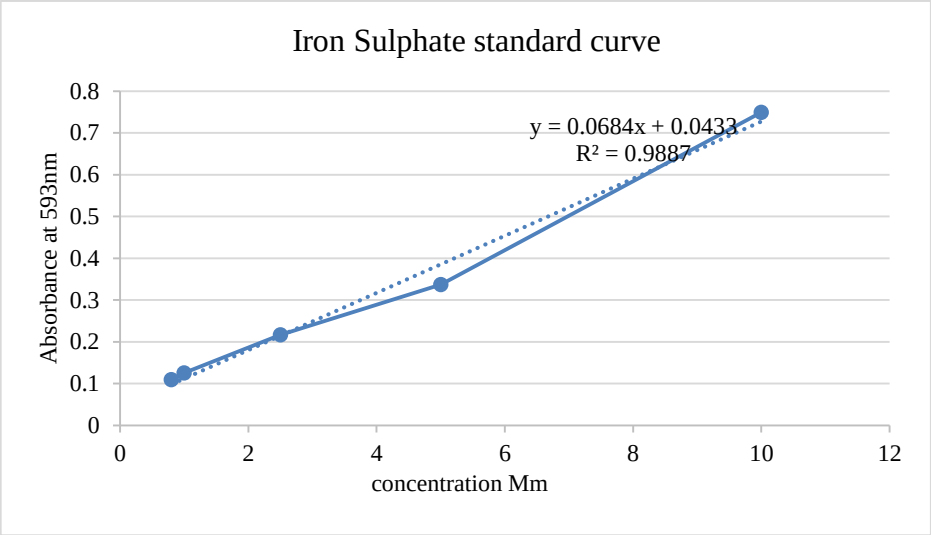

Figure 5.15. Iron sulphate calibration standard curve.

Table 5.2. The antioxidant activity of ascorbic acid, non-heated, roasted and boiled black seed mix. Values are means ± standard deviation. Means in the same column not followed by the same superscript are significantly different ($p< 0.05$).

Sample	Antioxidant capacity (mmol/g)
non-heat	9.9±0.02a
Roasted	9.8±0.02a
boiled	8.2±0.02b
Ascorbic Acid	6.3±0.00c
Water	$0.39±0.00_d$

Table 5.2 shows a significant reducing power for all black seed mix samples, even higher than that of the ascorbic acid control. It is assumed that higher concentration of ascorbic acid will present higher anti -oxidant power. However, no significant difference in reducing power is evident between non heated and roasted samples (9.9±0.02 versus 9.8±0.02 mmol/g). The results can be compared to that of Cho *et al*. (2014) who reported

no difference in reducing powder of non-heated and medium roasted coffee beans (5.98 versus 6.12 mmol/g). The authors reported a similar discrepancy between the FRAP and DPPH assay showing a significant increase in DPPH radical scavenging activity of the roasted coffee beans compared to the non- heated control, but no increase in reducing power.

The present results also show a significant decrease in reducing powder of boiled black seed mix, yet the value was still higher than that of the ascorbic acid positive control. The reason could be due the reduction of third stage MRPs in the boiled sample (compared to the roasted sample) that also play a role in reducing activity.

On the other hand, Vanghani and Van Wyk, 2013 and Gu *et al*., 2010 reported a 0.3 to 6 fold increase reducing power of MRP products compared to the non-heated components in casein – glucose and lysine- fructose model systems. There appears to be a discrepancy between reducing power of MRPs in model lysine reducing sugar systems and complex food systems. The explanation could be that complex seed containing black seed mix contains phenolic compounds that are susceptible to heat treatment which counteracts the positive effects of MRPs observed in model systems.

Whereas many published studies test a range of sample concentrations to determine anti -oxidant properties of plant extracts for example, studies undertaken to compare antioxidant properties of MRPs test only single concentrations of each sample. This approach is followed was followed in publications of Vanghani and Van Wyk (2013), Morales and Jimenez-Perez, (2001) and Cho *et al.,* (2014) who tested only single sample concentrations (the same concentration) for comparative purposes. The black seed mix extracts used in this study were all adjusted to the same soluble protein concentration as determined by the Bradford assay.

5.8.6.3 FTC and TBA methods

The antioxidant activities of extracts of the black seed mix heat treated with different methods were measured by the FTC method and compared with the results of the TBA method. In the FTC method, the peroxides formed during the initial stages of lipid oxidation were measured. As oxidation proceeds, peroxides are gradually decomposed into lower molecular compounds and measured with TBA reagent.

Table 5.3. Antioxidant activity of samples as measured by the FTC method after incubation for 8 days.

Sample	Absorbance at 500 nm	Inhibition %
Control	$1.65\pm 0.026_a$	0
BHT	$0.006 \pm 0.002_b$	99.6
non- heated sample	$0.688 \pm 0.022_c$	58.3
roasted sample	$1.614 \pm 0.014_d$	2.42
boiled sample	$0.577 \pm 0.034_e$	65.0

Inhibition % (capacity to inhibit the peroxide formation in linoleic acid). A high inhibition % indicates a high antioxidant activity. Control was incubated with linoleic acid but without the samples. Values are means ± standard deviation. Means in the same column not followed by the same superscript are significantly different ($p< 0.05$).

The results show a significant lipid antioxidant ability of the non-heated black seed mix (66%) which was increased to 71% by boiling of the sample. However, the roasted sample showed significantly lower anti- oxidant ability (19%). Table 5.3 and Table 5.4 shows the antioxidative activities measured using TBA, which shows a similar to that of the FTC method. The results are comparable to that of Zainol (2003) reported an ability of 40-60% of a Malaysian herb using the FTC and TBA method. The authors correlated the antioxidant activity with the phenolic content in the herb.

Table 5.4. Absorbance of TBA treated samples after 8 days of incubation using the FTC test. Control was incubated with linoleic acid but without the samples. Values are means ± standard deviation. Means in the same column not followed by the same superscript are significantly different ($p< 0.05$).

Sample	Absorbance at 532 nm
Control	$1.995\pm 0.0200_{a}$
BHT	$0.006\pm0.0004_{b}$
non- heated sample	$0.303\pm0.0094_{c}$
roasted sample	$0.496\pm0.0047_{d}$
boiled sample	$0.436\pm0.0094_{e}$

The results in Table 5.3 show a significant lipid antioxidant ability of the non-heated black seed mix (66%) which increased to 71% following boiling of the sample. Table 5.4 shows the antioxidative activities measured using TBA, which are similar to that of the FTC method The results are comparable to that of Zainol (2003) who reported an antioxidant ability of 40-60% of a Malaysian herb using the FTC and TBA method. The authors correlated the antioxidant activity with the phenolic content in the herb.

However, the roasted sample showed significantly reduced ability to inhibit of lipid peroxidation (19%) compared to the control. The decrease in is comparable to decrease in antioxidant activity measured by FRAP and DPPH of darkly roasted coffee beans compared to lightly roasted coffee beans as reported by Cho *et al.*, (2014). The authors correlated the decrease with a loss in phenolic content. Similarly, Morales and Jimenez-Perez (2001) reported a significant loss in anti-oxidant activity of lysine-reducing sugar complex that was heat treated extensively (late stage MRPs), whereas the intermediate stage MRPs demonstrated significantly higher anti -oxidant activity than the non -heated control. These tests were done with the FRAP and DPPH assay, but not the FTC and TBA assays. The conclusion reached is that roasting of the black seed mix could have damaged the phenolic compounds to a certain degree and that the increase in third

stage MRPs could also have diminished its power to inhibit lipid oxidation. The results reported here confirm this phenomenon for the first time using the FTC and TBA assays.

5.9 Discussion

The results in this chapter show that the non- heated black seed mix contain significant antioxidant properties including radical scavenging, ferric reducing and inhibition of lipid oxidation properties These properties could be attributed to the phenolic compounds in the black seed mix present in fenugreek (Akbari *et al*, 2012), sesame oil (Namiki, 1995), millet (Chandrasekara and Shahidi 2011) and treacle (Rothwell *et al*., 2013).

Roasting of the black seed mix resulted in increased third stage MRPs, and associated increase in radical scavenging activity. This increase could be due to the radical scavenging effect of third stage MRPs (as demonstrated in model MRP systems) as well as release of phenolic compounds from seeds in the black seed mix similar to that reported for light and medium roasted coffee beans Cho *et al*., (2014). However, the ferric reducing power remained similar to that of the non -heated black seed mix. The ability to reduce lipid peroxidation was significantly reduced, which could be due to damage of the phenolic compounds caused by too much heating of the black seed mix, Reduction of radical scavenging activity was observed in darkly roasted coffee beans. It is speculated that there is a balance between antioxidant benefits of MRPs and damaging of phenolic compounds by roasting process that would affect different anti oxidation mechanisms differently.

Boiling of the black seed mix resulted in increased second stage MRPs and reduced third stage MRPs. This correlated with increased radical scavenging activity, relatively little decrease (although significant) in ferric reducing activity and increased inhibition of lipid peroxidation ability. The latter was similar to that of a synthetic anti-oxidant added to foods.

The results reported here provide evidence for significant antioxidant properties of the non- heated black seed mix which is probably due to its high content of phenolic compounds in seeds and treacle.

We report here for the first time the difference in the inhibition of lipid oxidation by quantified intermediate-stage MRP (boiled) compared to quantified third-stage MRP (roasted) using the FTC and TBA assays in a plant seed containing food system. The third stage MRPs actually increased lipid peroxidation, which corresponds to increase in peroxide value of the roasted black seed mix after 2 month accelerated shelf life study (Figure 4.6 Chapter 4).

The present findings suggest that the boiled and roasted black seed mix contain high radical scavenging antioxidants. Currently, the radical scavenging activity of antioxidants that are naturally present in food products is regarded as a quality parameter to indicate the dietary value (Ak and Gulcin, 2008).

To our knowledge, the effects of quantified different stages of MRP as generated by different heating methods of a plant food system on antioxidant properties have not been studied although many such studies have been reported on model systems of lysine and reducing sugar complexes (Wang *et al*., 2011, Chawla *et al*., 2009, Maillard *et al*.,2007, Amarowicz, 2009, Gu *et al*., 2010). Whilst these studies might be relevant to the dairy and egg industry, it is less relevant to plant containing foods where phenolic compounds also play a role in antioxidant activity, and might be destroyed by too harsh heating conditions.

Further research is needed to establish the antioxidant mechanisms in heat treated plant containing foods, as the relation between phenolic, or other anti-oxidants and MRPs appears to play a key role in the overall nutritional outcome.

CHAPTER 6

Effect of Different Processing Conditions and the Addition of Additives on the Immune Reactivity of the Black Seed Mix

6.1 Introduction

6.1.1 Lay out of the chapter

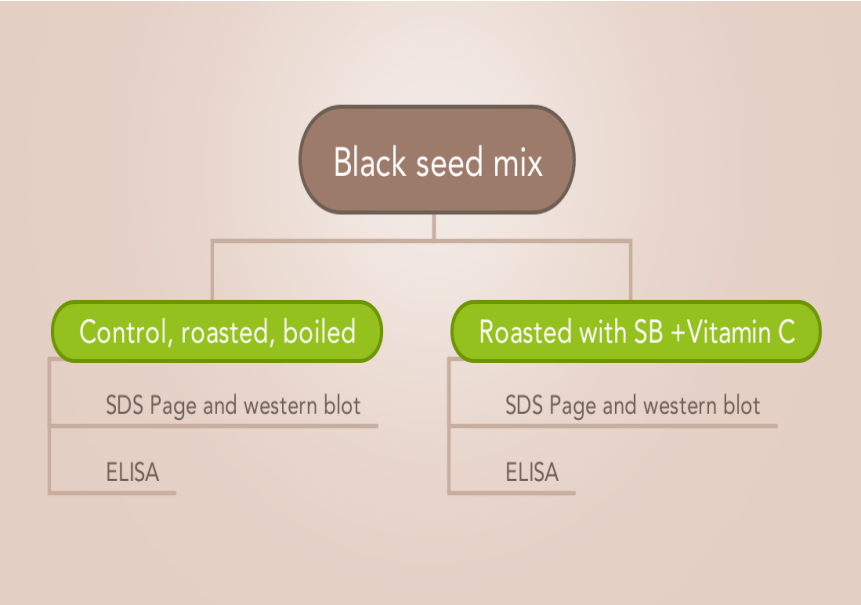

Figure 6.1. Lay out of the chapter.

6.1.2 Aims of the study

1. To measure the effect of different thermal processing methods on the composition, solubility, structure, and immune reactivity of peanut allergens extracted from the product.
2. To test the effect of additives such as sodium bisulphite and ascorbic acid on the structure and immune reactivity of peanut proteins.

6.1.3 Novelty of the study

1. Study of the effect of different thermal processing methods of the black seed mix on peanut immune reactivity. Previous studies on the immune reactivity of glycated peanut proteins have been carried out on isolated peanut proteins or on peanut-sugar or peanut-polysaccharide mixtures. There are no reports on the immune reactivity of a mixture of proteins glycated in a complex low water food matrix.

2. The effect of the addition of sodium bisulphite or ascorbic acid to a complex food matrix on peanut protein immune reactivity and structure has not been studied. A study was recently published that reports the effect of sulphite allergens isolated from cashew nuts.

6.1.4 The allergenic response

Some foods can provoke adverse reactions in some people. There are two main types of adverse reactions, those caused by toxins in the food and negative reactions resulting from an allergy to a component of the food. Food allergies are defined by the European Academy of Allergology and Clinical Immunology (EAACI) (Besler, 2001). There are two subcategories of food allergic reaction. First is non-IgE mediated allergies, which result from an interaction between cells and a chemical mediator, rather than antibodies; the symptoms of such a reaction occur within the first hours or the first day after exposure to the food, and the reaction can lead to symptoms in different parts of the body, such as the skin, gut, and other organs (Taylor *et al.*, 2002). The second subcategory is IgE mediated allergies, which are caused by the production of antibodies (immunoglobulin E) and their interaction between various cell types and chemical mediators. The symptoms of such reactions can be found in the mouth, gut, and skin (Taylor and Hefle, 2002).

There are five structures of immunoglobulin classes in human antibodies (IgA, IgD, IgG, IgE, and IgM). The molecular structures of various immunoglobulins (IgEs) are an integral part of the immediate allergic response. An allergic condition occurs when an allergic person consumes certain foods. These foods stimulate the immune system to produce IgE specific to allergic epitopes in food. The IgE molecule is found on immune system cells including basophils and mast cells. The two cells are very important in allergic reactions. Basophils are phagocytic white cells that engulf foreign bodies in the bloodstream (Figure 6.2). When antibodies in both basophils and mast cells come into

contact with the food material, the cells stimulate the synthesis of hormones such as histamine, prostaglandins, and leukotrienes. These compounds cause allergic reactions in the body (Scaravelli, 2015). T lymphocytes are the other cell types that can be affected in allergic mechanisms by the presence of the allergen and release mediators (IL-3, IL-5, GM-CSF, IL-4, and IL-13). These T cells in turn stimulate B cells to produce IgE (Scaravelli, 2015). The mediators released by the mast cells lead to the typical allergic symptoms, such as nausea, vomiting or diarrhoea, and skin symptoms, including eczema.

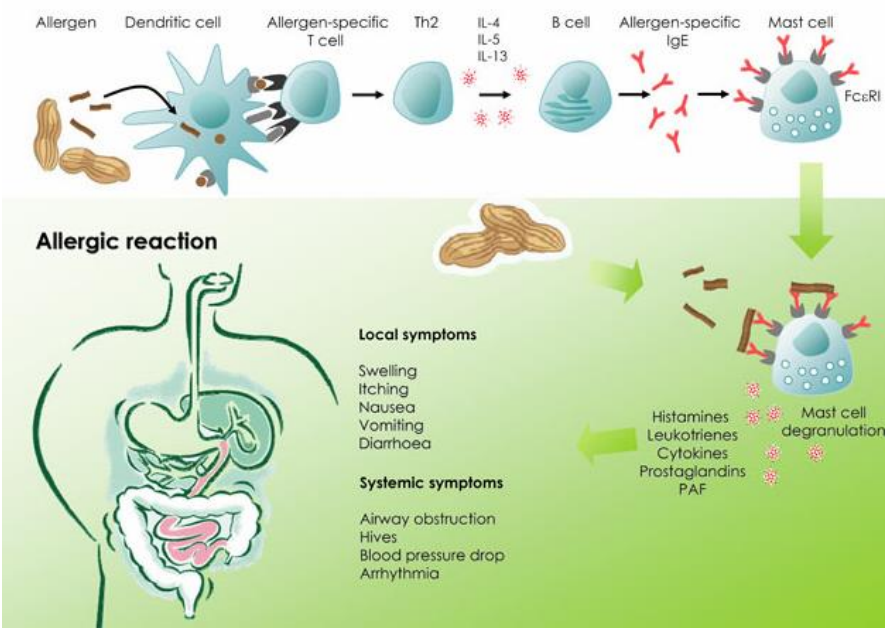

Figure 6.2. Mechanism of an IgE-mediated allergic reaction (Ucfa, 2014).

Usually, food allergens are proteins or glycoproteins between about 10 and 70 kDa in molecular mass. They originate from different protein families, but only a small percentage of proteins are allergenic (Scaravelli, 2015).

6.1.5 Classification and structure of allergenic plant proteins

Plant tissues contain vast numbers of proteins, which can be classified on the basis of their physical properties (e.g., solubility), functions (storage, structural, metabolic, or protective), or structural and evolutionary relationships (Shewry *et al.*, 2012; Breiteder and

Ebner, 2001; Mills *et al.*, 2003). As the focus of this study is on allergenic proteins, we chose to discuss the proteins based on allergenicity according to a literature review. Amongst the wide range of plant food proteins consumed by humans, a majority of plant food allergens fall into two plant protein families: the cupin superfamily and the prolamin superfamily (Shewry *et al*., 2012).

6.1.6 The cupin superfamily

The cupin superfamily includes a wide range of proteins, including globulin storage proteins from seeds, with 7S and 11S seed globulins being major allergens in soybeans and peanuts. Globulins are classified as such because of their solubility in salt solutions.

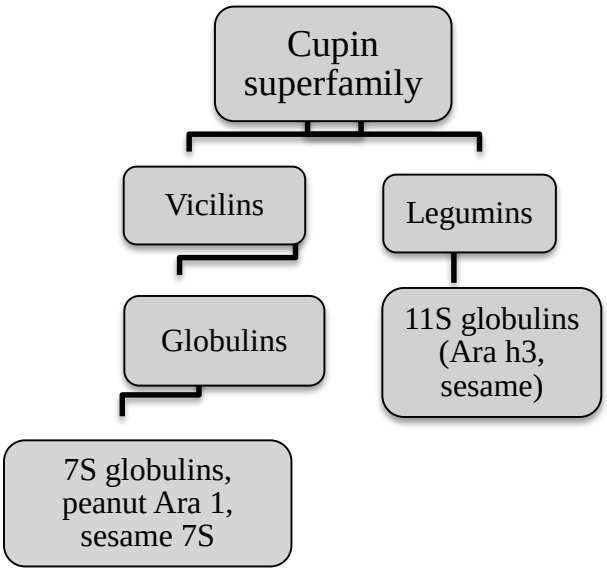

Figure 6.3. The cupin superfamily.

Figure 6.3 shows a schematic diagram of the cupin superfamily. The cupin superfamily is based on a core conserved beta barrel structure called the cupin domain. The legumin and vicilin have similar structures and are based on subunits assembled to form trimers and hexamers (Shewryet *et al*., 2012). Vicillins are the most common legume-derived globulin protein in the human diet and consists of two cupin domains (Figure 6.4

B). The collective name in legumes is 7S globulins which in peanuts are called peanut conarachin or Ara 1 (Murzin *et al*., 1995). Globulin proteins are the prevailing storage proteins in legume seeds, and it accounts for 50-90% of seed proteins (Mills *et al*., 2009). The legumin sub-groups of the cupin family contain the 11S globulin (collective name in legumins), which is called arachin h3 in peanuts (Figure 6.4 C and E). 11S globulins are hexameric proteins that are transported through the secretory system as intermediate trimers. These storage proteins contain acidic or basic α- and β-polypeptide chains that are linked by a disulphide bond between cysteine residues in the basic and acidic chains (Shewry *et al.*, 2012).

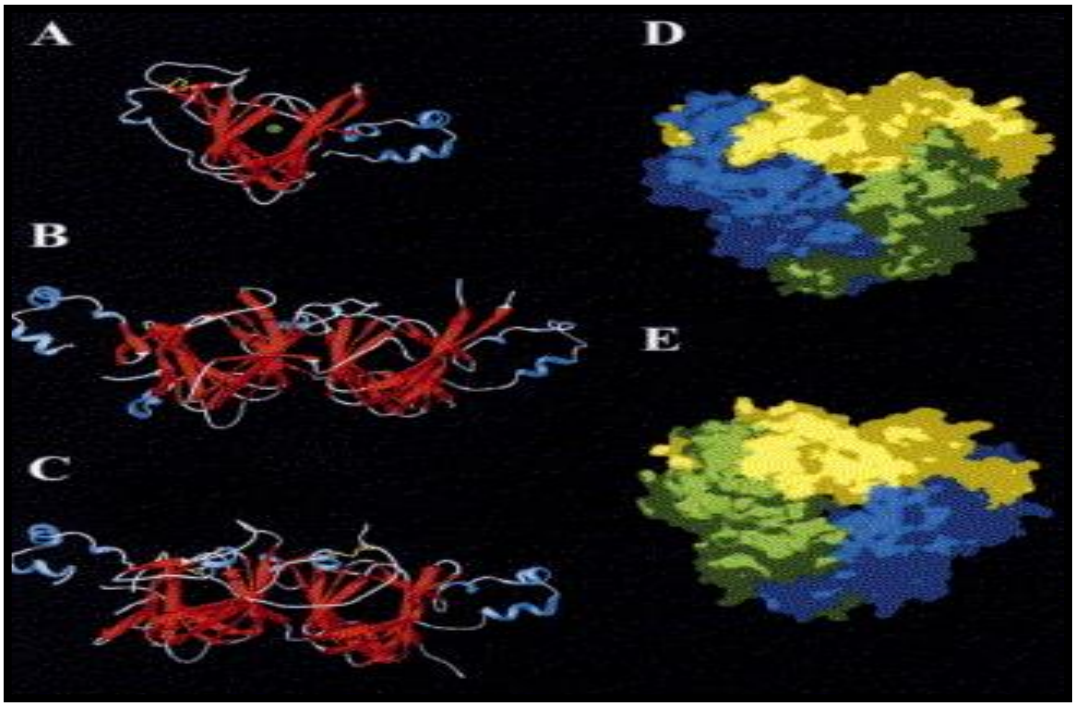

Figure 6.4. Protein structures of the soybean-derived cupin superfamily (Breitender and Radauer, 2004).

Image A shows a protein that contains one cupin domain. Image B shows vicillin that consists of two cupin domains. Image C displays a globulin protein in the legumin sub group. Images D and E are the molecular surfaces of vicillin and legumin. The yellow areas

in the images are the disulphide bonds, the blue are the α-helices, the green are the manganese ions, and the red are the β-barrel structures (Breitender and Radauer, 2004).

6.1.7 The prolamin superfamily

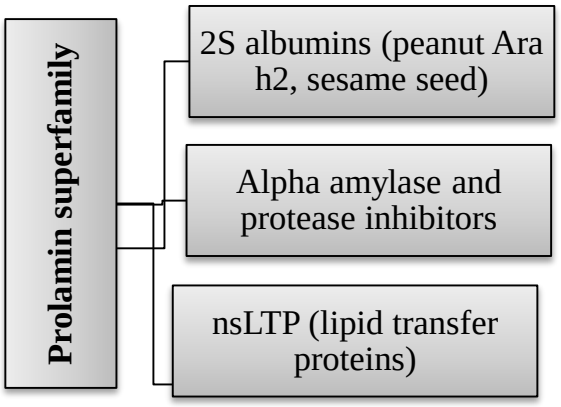

Figure 6.5. Schematic diagram of the prolamin superfamily.

The prolamin superfamily includes the major storage proteins of cereal grain (Figure 6.5). They include three groups of proteins that have similar three-dimensional structures that are rich in a-helix and account for about half of the characterised plant food allergens: 1) 2s albumin seed storage proteins, 2) non-specific lipid transfer proteins from a range of plant tissues, and 3) inhibitors of *a*-amylase and trypsin from cereal seeds. The prolamin family is characterised by a high content of glutamin. It includes 2S albumin (Figure 6.6 A), the major allergen in some nuts and seeds. 2S albumins are part of a heterodimeric protein that contains two polypeptide chains that are that linked by disulphide bonds (Shewry *et al.*, 2012).

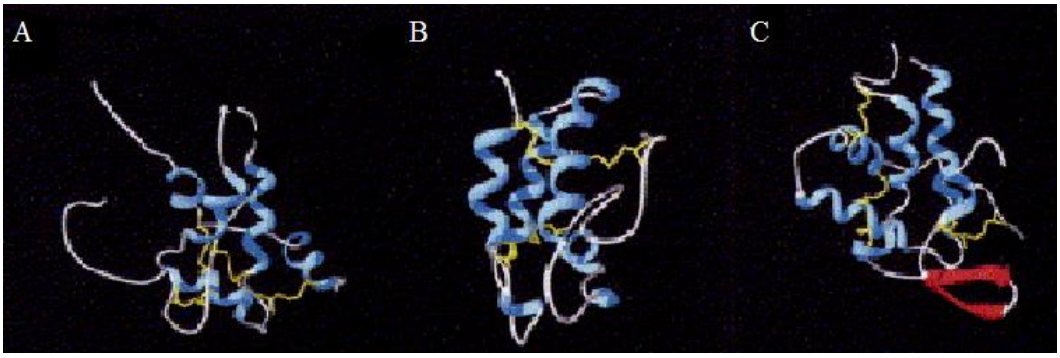

Figure 6.6. Protein structures of the prolamin superfamily. A is 2S albumin, B is nsLTPs, and C is a wheat α-amylase inhibitor. The yellow represents disulphide bonds, the blue α-helices, and the red β-strands (Breitender and Radauer, 2004).

The major proteins in the black seed mix are derived from peanut (2.99%, Chapter 3 Table 3.3) and sesame (2.55% Chapter 3 Table 3.3), so the next section will address the properties of these proteins.

6.1.8 Peanut protein

Peanut contains about 26% protein, consisting of albumen and globulin. The globulin contains about 96% of the total protein and consists of two groups: arachin and conarachin. The conarachin accounts for about 33%, with arachin comprising about 63% of the total protein (Figure 6.7) (Chun, 2002).

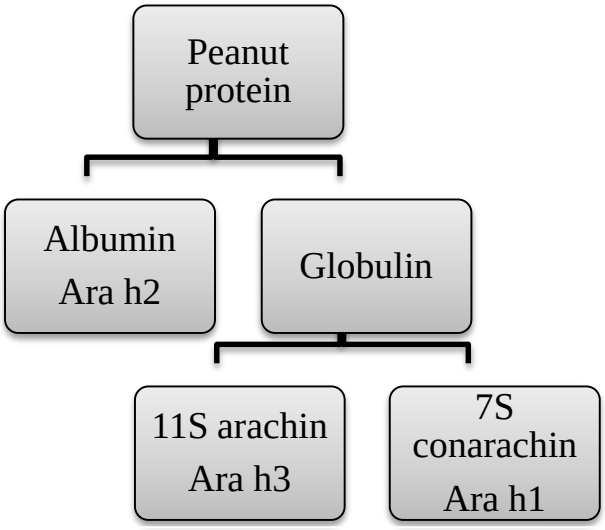

Figure 6.7. Subfractions of peanut protein.

Figure 6.8 shows the gel electrophoresis for arachin and conarachin in peanut protein. The arachin (Ara h3) 11S globulin (vicillin) consists of three acidic (α) subunits with molecular weights of 42.0, 39.0, and 35.0 kDa and a basic (β) subunit with a molecular weight of 22.0 kDa held together by disulphide bonds (Zhao *et al.*, 2011; Feng *et al*., 2014),. The conarachin (Ara 1) 7S globulin (vicillin) consists of one subunit with a molecular weight of 64.0 kDa (Hu *et al.*, 2011). Arachin is rich in sulphur but poor in phenylalanine and tyrosine, whereas conarachin is poor in sulphur, lysine, and methionine but rich in threonine and proline (Quist, 2005).

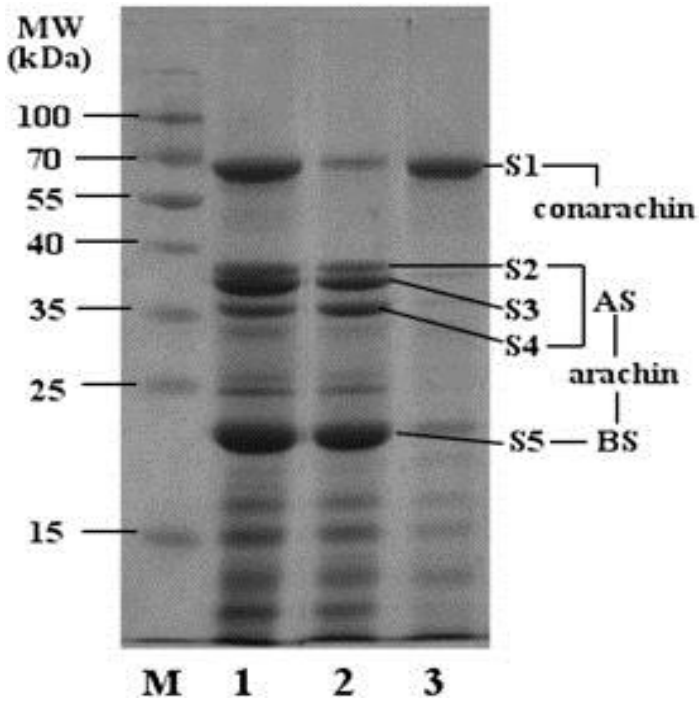

Figure 6.8. SDS-PAGE profile (under reducing conditions) of crude peanut protein extracted with 0.3M phosphate buffer at pH 7.4 (lane 1, 2 and **3**, arachin (P7S) and conarachin-rich (7S) extract (Feng et al., 2014).

Ara h2 is an albumin classified as such because of its high solubility in water. It is not visible on the gel above because of its low concentration (4% of total protein); it is composed of two polypeptides of approximately 17 kDa, which have essentially similar amino acid sequences Ara h2 proteins form four intermolecular disulphide bridges, which stabilise the structure of the protein (Burks *et al.*, 1992).

6.1.9 Sesame protein

Most of the proteins present in sesame seeds are storage proteins such as albumins (8.9%), globulins (67.3%), prolamins (1.3%), and glutelins (6.9%) on the basis of their solubility. The water-insoluble sesame 11S globulin constitutes 70-80% of the total seed proteins in sesame (Orrunoo and Morgan, 2007). The 7S globulin constitutes approximately 5% of the total sesame protein, whereas the peanut 7S (Ara 1) constitutes 33% of the total protein.

The water-soluble sesame 2S albumin constitutes 8.9% (Orrunoo and Morgan, 2007) of the sesame protein, whereas the peanut 2S constitutes only 4%.

6.1.10 Effect of thermal treatment on protein structural properties

Proteins are sensitive to temperature which means they are denatured at high temperature. Protein denaturation is commonly defined as any change of original native structure of a protein which does not alter the sequence of amino acids. Denaturation takes place for the reason that the bonding interactions accountable for the secondary and tertiary structure are disrupted; these comprise hydrogen bonds and electrostatic interactions, hydrophobic interactions, ionic bonds, and covalent bonds (Cramp, 2007). Whereas most types of bonds can be disrupted by temperature, covalent bonds are strong chemical bonds that may break and form under appropriate thermal conditions, such as the disulphide bonds between 11S acidic and basic subunits in soy protein (Wolf, 1993). Identification of disulphide bonds can be performed by dispersing protein samples in solvent including 0.2M 2-Mercaptoethanol or dithiothreitol (DTT), which reduces disulphide bonds to sulfhydryl groups (Zhong *et al*., 2006). Sulphites was also shown to disrupt disulphit bonds in proteins (Cecil and Wake, 1962)

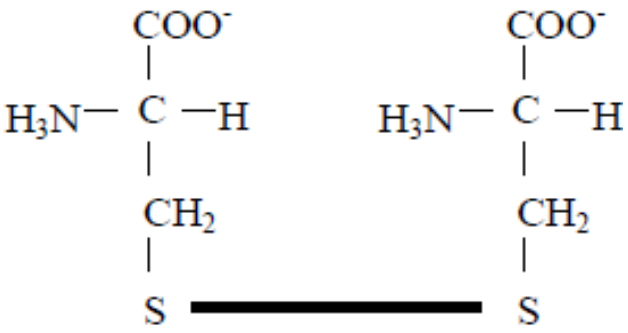

Figure 6.9. Covalent disulphide bonds between two cysteine residues (Wolf, 1993).

Thermal treatment of Table 6.1 shows that the heat in the stability of allergens is associated with the presence of sulfhydryl groups or disulphide bonds in the protein structure.

Table 6.1. Presence of sulfhydryl and disulphide groups of plant proteins (Barre et al., 2005).

Protein Family	Presence of Sulfhydryl and Disulphide Groups
7S	Lack of SH groups
11S	Subunits are linked with disulphide bonds
2S albumins	4 SH groups

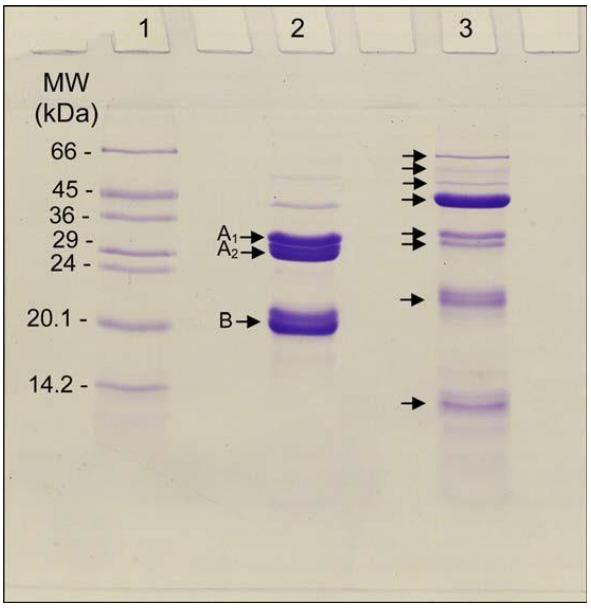

Figure 6.10. SDS-PAGE of 11S and 7S sesame globulin under reducing conditions. Lane 1: low-range molecular weight markers; Lane 2: sesame 11S globulin; Lane 3: sesame 7S globulin. A1 and A2: acidic polypeptides; B: basic polypeptides; the main subunits of the 7S globulin are indicated by arrows (Orrunoo and Morgan, 2007).

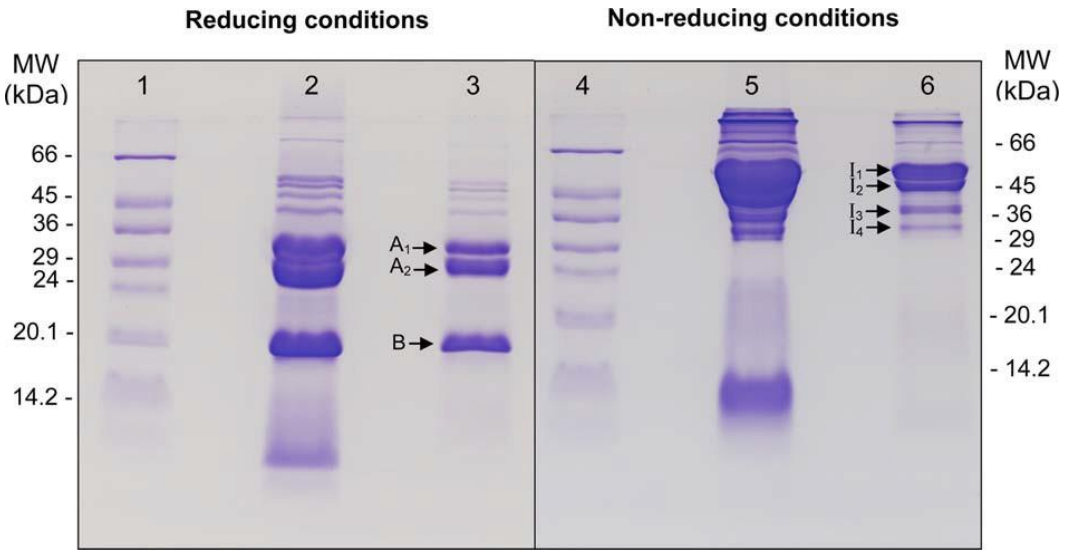

Figure 6.11. SDS-PAGE of extract of sesame seeds and purified 11S globulin samples under reducing and non-reducing conditions. Samples were run on 15% acrylamide gels. Lanes 1 and 4: low-range molecular weight markers; Lanes 2 and 5: ammonium sulphate extract of sesame seeds, further purified by column chromatography. Samples shown in lanes 1, 2, and 3 were treated with reducing agent dithiothreitol (DTT). Samples shown in lanes 4, 5, and 6 were not treated with DTT. A1 and A2: acidic polypeptides; B: basic polypeptide; I1, I2, I3, and I4, intermediary subunits (Orrunoo and Morgan, 2007).

Table 6.2. Comparison of composition of peanut and sesame seed protein based on molecular weight by SDS PAGE (Barre *et al*., 2005).

	Peanut		**Sesame**	
	%	**Molecular weight kDa***	**%**	**Molecular weight kDa***
11S globulin	63	α subunits 42, 39, and 35; β subunit 22	80	α subunits 30 and 24; β subunit 20
7S globulin	33	64	5	45
2S albumin	4	17	8.9	14

**Molecular weight at reducing conditions*

When focusing on globulin storage proteins, research shows that these proteins have some common properties when they are treated with high temperature. For example, when heated, they tend to form large thermally induced aggregates. Thus 7 S and 11 S globulins starts to denature when heated to a temperature of around 70-75 °C. As studied by the differential calorimetry (Mills *et al.*, 2003). Conformational studies by Fourier

transform infrared spectroscopy of soy vicilin (7S globulin) showed that the cupin beta barrel structure remained intact during heat treatment, although aggregates were formed, which could explain conserving of allergenic properties even after heat treatment (Mills *et al.,* 2003).

6.1.11 Allergenicity of peanut proteins

Food allergies may be the most significant allergy resulting in clinical problems, and of these allergies, peanut allergy is perhaps the most serious. Peanut allergy is typically lifelong, and sensitive individuals can experience symptoms ranging from mild to life-threatening anaphylaxis. Food anaphylaxis fatality records report peanuts as the cause of a majority of the deaths attributed to food allergies over the last five to seven years (Block *et al*., 2007). Peanut allergy is the most common food allergy in children and adults, and, in recent years, peanut allergies have increased and are estimated to affect between 0.6% and 1% of the US and EU populations (Mondoulet *et al.,* 2005). The allergenic proteins present in peanuts are Ara 1, Ara 2, and Ara 3 (Watson, 1998; Koppelman *et al*., 2003). IgE molecules are the trigger of the immune response to allergens. Many studies have focused on identification of IgE binding epitopes in proteins, which are also called B-cell receptors. B-cell epitopes refer to antigenic determinants and are very critical in the development of peptide-based vaccines. There are two categories of B-cell epitopes, including continuous and discontinuous. In linear B-cell epitopes, the amino acids are in consecutive order while in discontinuous epitopes; the amino acids are spatially folded such that they are away from the primary sequence. Health practitioner or researchers developing antibodies or performing immunodiagnostic rely on linear B-cell epitopes because they are easy to determine. (Singh *et al.* 2013). Linear epitopes are comprised of a single sequential part of the primary amino acid sequence of the protein. Conformational epitopes comprise multiple amino acid sequences, which are brought together spatially by

the protein's three-dimensional structure (Barre *et al*., 2005). Conformational epitopes, as they are dependent on a protein's three-dimensional structure, are less stable against structural changes brought about by heat compared to linear epitopes, which are not affected by conformational changes. Linear epitopes have been found to play the most important role as IgE binding sites in food allergies. Several studies have shown that Ara 1, Ara 2, and Ara 3 are the main allergenic proteins of the peanut (Barre *et al.*, 2005). Ara 1 and Ara 2 are recognised by serum IgE in >90% of peanut allergy patients, indicating that they comprise a majority of peanut allergens (Rabjohn *et al.* 1999). Ara 1 and Ara 2 contain linear IgE-binding epitopes (Barre *et al.*, 2005).

6.1.11.1 Conarachin (ARA H1), 7S globulin peanut protein

Ara h1 is a 63 kDa glycoprotein that is reported to occur in peanuts as a highly structured, stable trimer (Shin *et al.*, 1998; Koppelman *et al.*, 1999). Throughout the complete amino acid backbone of the protein, 24 linear epitopes have been mapped (Burks *et al.*, 1997).

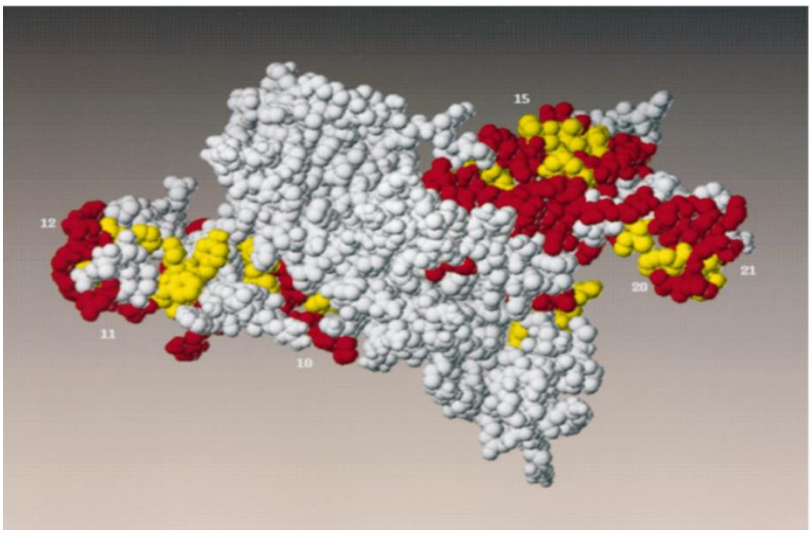

Figure 6.12. Structure of Ara h1 , where the red areas represent the IgE-binding epitopes from 10 to 22 epitopes, and the yellow areas are critical for IgE binding to occur (Shin et al., 1998).

6.1.11.2 ARA H2, 2S albumin peanut protein

Ten epitopes have been identified on Ara h2 (Barre *et al*., 2005)

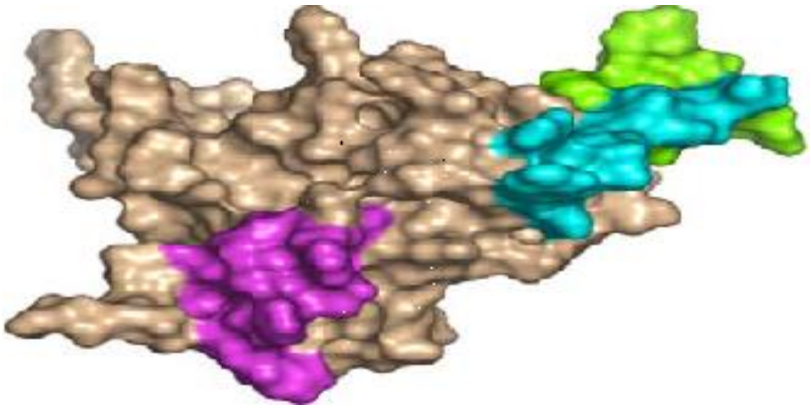

Figure 6.13. Structure of Ara h2 , where the purple, green, and blue areas are the majority of B epitopes (Barre *et al*., 2005)

6.1.11.3 Arachin (Ara h3), 11 S globulin peanut protein

Ara h3 has four IgE binding epitopes, all situated on the acidic polypeptide of the allergen (Rabjohn *et al.*, 1999). IgE binding to the basic polypeptides of Ara h3 has also been reported, which indicates the presence of more IgE binding epitopes than the four reported ones Table 6.3 (Koppelman *et al.*, 2003).

Table 6.3. The Allergenic epitopes of the protein (Barre *et al*., 2005).

Protein Family	Allergenic Epitopes
Peanut Ara 1	5 immunodominant epitopes
11S peanut Ara 3	Linear lgE binding, 4 on acidic polypeptide
2S albumins	10 linear lgE-binding epitopes
Peanut Ara 2, 6, and 7/sesame seeds	10 epitopes (majority of B-cell epitopes)

6.1.12 Effect of thermal processing on peanut proteinimmune reactivity

Despite the high levels of allergenic proteins in peanuts, there are variations in the number of individuals exhibiting allergic reactions to peanuts across different countries. This

difference can be attributed to the use of different food preparation and cooking techniques. For example, sensitisation and reactivity to peanuts is less prevalent in China than in the United States, where traditional cooking methods are very used. Research has indicated that the allergenic properties of peanuts are significantly affected by the preparation methods employed, with roasting resulting in a far higher level of allergenic properties than boiling or frying (Beyer *et al.,* 2001). Recently, Soares -Weiser *et al.,* (2014) demonstrated a reduced allergenic response to boiled peanuts by the skin prick test in humans.

Heat treatment of proteins in the presence of reducing sugars can cause covalent modification, involved in glycation and Maillard rearrangement. According to previous research, the IgE binding to Ara h1 and Ara h2 purified from peanuts was increased upon heat treatment in the presence of reducing sugars (Barre *et al.,* 2005). The explanation was that heating causes structural changes that lead to Ara h1 oligomerisation, leading to increased stability against the gastric digestion of peanut allergens. Another study using recombinant Ara h2 showed similar effects of the Maillard reaction on IgE binding (Barre *et al.,* 2005). However, the effect of the stages of Maillard reaction products to increased IgE binding were not investigated in these studies

6.1.13 Effect of reducing or oxidation agents on peanut allergenicity

Amongst other structural changes of peanut proteins caused by thermal treatment, disulphide exchange plays a large role. Although the effect of reducing agents such as mercaptoethanol by Western blotting showed no difference between reduced and non-reduced antigen reactivity (Barre *et al.*,2005), probably because conformational epitopes have been destroyed by requirement of boiling of samples and addition of SDS before electrophoresis, therefore immune reactivity will only be detected against linear epitopes. The effect of the addition of reducing or oxidation agents to proteins on IgE binding by

ELISA has not been investigated. Conformational epitopes of samples used for ELISA testing will not be affected because samples are dissolved in phosphate buffered saline and do not require addition of SDS or boiling before analysis. We postulate that the addition of food grade oxidation or reducing agents to the black seed mix could affect the immune reactivity of proteins because of the prevalence of disulphide bonds in 11S and sulfhydryl groups in 2S allergens.

Zhang and Sun (2008) report the breaking of the disulphide bonds of the subunits by addition of the reducing agent sodium bisulphite, as well as increased hydrophobicity and increased denaturation temperature. Furthermore, Abtahi and Aminlari (1997) reported improved solubility of soybean milk proteins by addition of sodium sulphite, sodium dodecyl sulphate and cysteine have been used to improve the protein solubility of soy products However, and neither of the research groups investigated the effect on immune reactivity of proteins. A recent article by Mattison *et al*. (2014) reports the disruption of disulphide bonds of allergens isolated from cashew nuts by addition of sodium sulfite with an associated reduction of IgE in cashew-allergic patients as determined by the Western blotting and ELISA techniques. Sulphite has been shown to disrupt intra- and inter-chain disulphide bonds of several proteins (Cecil and Wake, 1962).

On the other hand, vitamin C (ascorbic acid) accelerates the formation of disulphide bonds in heat-treated food proteins (Gaonkar and McPherson, 2006) however the effect on immune reactivity has not been reported. We aim to investigate the effect of the addition of sodium bisulphite and ascorbic acid to the black seed mix on the immune reactivity of peanut proteins.

Sodium bisulphite is a food additive, E222, and is used as food preservative in wine, fruit squash, jams, and frozen shellfish. The maximum limit of addition to cinnamon sticks is 150 mg/kg (Commission Directive 2010/69/EU). Sulphite-containing

compounds, including sodium sulphite (GRAS 182.3798), sodium bisulphite (GRAS 182.3739), and sodium metabisulphite (GRAS 182.3766) are on the FDA's GRAS list. Generally recognised as safe (GRAS) compounds, when used at the minimum amount required for the intended purpose in human foods and cosmetics, are considered safe by the US Food and Drug Administration (FDA). Sulphites are multi-purpose compounds commonly used as calcium, potassium, or sodium salts in the food and pharmaceutical industries as preservatives or antioxidants. Although they are generally considered safe, sulphites can cause contact dermatitis in cosmetics in rare cases. Vitamin C or ascorbic acid has an E number of E-300 and is widely used as an antioxidant in foods.

6.2 Principles of the methods used in the chapter

6.2.1 SDS-PAGE (polyacrylamide gel electrophoresis)

SDS-PAGE is a technique used by analysts to separate molecules based on size. The principle behind this method is that charged molecules will move towards an electrode with an opposite charge. Electrophoresis technique cannot be used to determine the size of molecules in gel form. However, this is because the movement of molecules in gel form depends on both size and charge. Therefore, to overcome this hindrance, the sample needed to be treated such that all the molecules have a uniform charge and, therefore, the movement of the molecules depends solely on size. To produce uniformly charged molecules, the proteins molecule needed to be denatured using SDS. Protein denaturing ensures that the protein molecule unfold and lose their tertiary structure. As a result of using SDS, all the protein molecules are negatively charged and when the gel is placed in electric field, the molecules will migrate to the anode, whereby, the molecules can be separated based on size using molecular sieving effects. This can be followed by visualization using appropriate staining methods and the size of the protein detected using known molecular weight marker (Walker, 2002).

6.2.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a testing method commonly used in food industry and official food control agencies. The method is used to test allergens in food. The method uses the principle of an enzyme linked to an antibody to detect the formation of a complex formed between the antibody and antigen. Polyclonal or monoclonal antibodies are used in this test. (As a result, the antigen in the food can be recognized by reading the label of the bound enzyme (Wieslab, 2000)

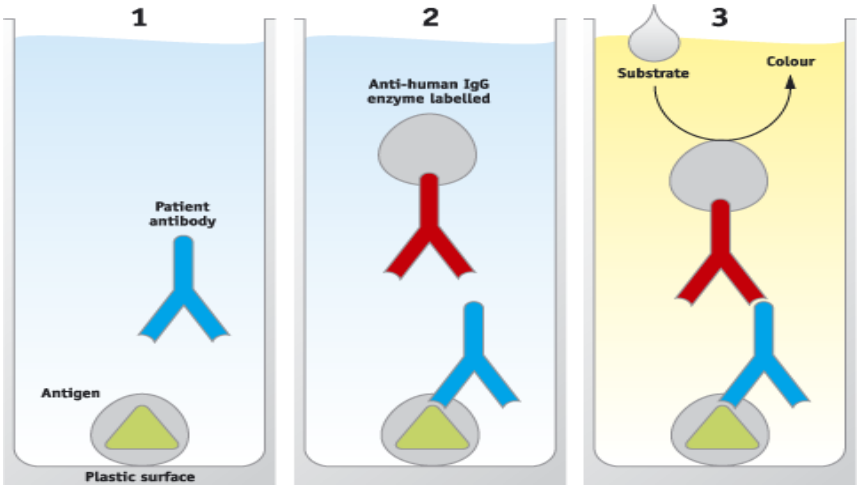

Figure 6.14. Principle of the direct ELISA method (Wieslab, 2000).

6.2.3 Western blot

The western blot technique is the commonest method used to investigate antigens. It uses the principle of a direct assay, which is the use of a known enzyme against the target antigen, for example, peanut (Moore, 2009).

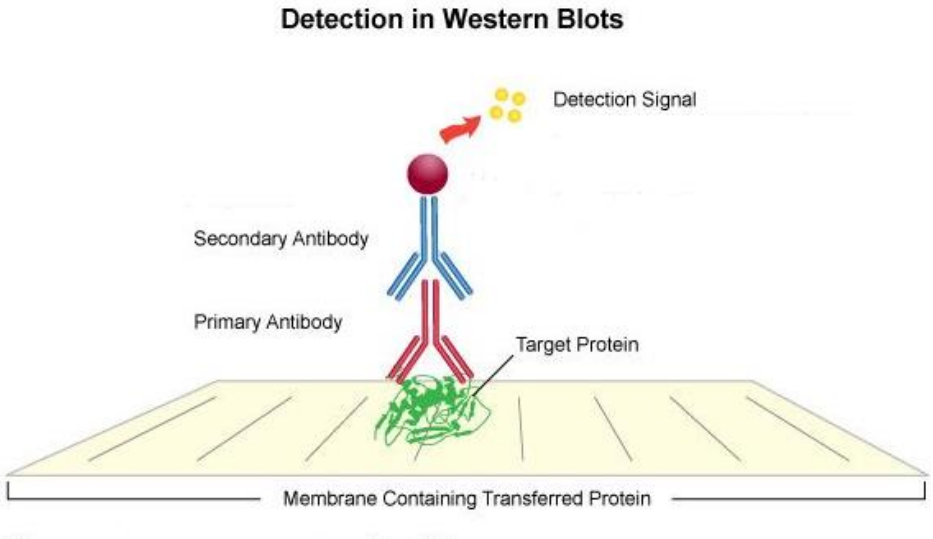

Figure 6.15. Detection of Western blots (Leince Technologies, 2006).

6.3 Materials and methods

The materials and methods used within this study were described in chapter 2 (2.2.11)

6.4 Results

6.4.1 Effect of different heating conditions on the immune reactivity

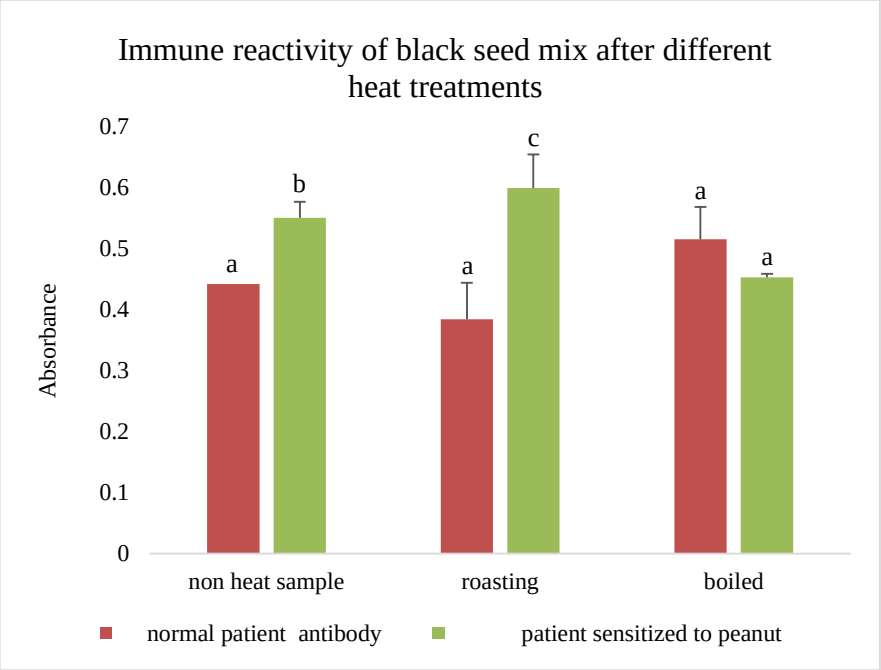

Figure 6.16. Immune reactivity as measured by ELISA of the black seed mix proteins subjected to different thermal treatments against the sera of patients allergic to peanuts. Heat treatments were non-heated, roasting (standard process), and boiling of peanuts and sesame seeds before addition to the black seed mix. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences ($P<0.05$).

The results in Figure 6.16 show that the immune reactivity of allergic patient serum increased significantly against the roasted black seed mix compared to that of the non-allergic patient's serum. However, allergic patient sera show no significant increase in reactivity against the non-heated and boiled black seed mix extract compared to the non-allergenic patient serum. These results correspond to that of Beyer *et al*., (2001) who reported that boiled peanuts have lower allergenicity in comparison with raw peanuts because the IgE binding to Ara h1, Ara h2, and Ara h3 is less in boiled peanuts.

6.4.2 Effect of protein digestion on immune reactivity

One of the characteristics of protein allergens is their ability to resist digestion (Cabanillas *et al*., 2012). Our results confirm this finding as the ELISA test show no significant difference in immune reactivity between digested and non-digested extracts of black seed mix prepared with different heating conditions (Figure 6.17).

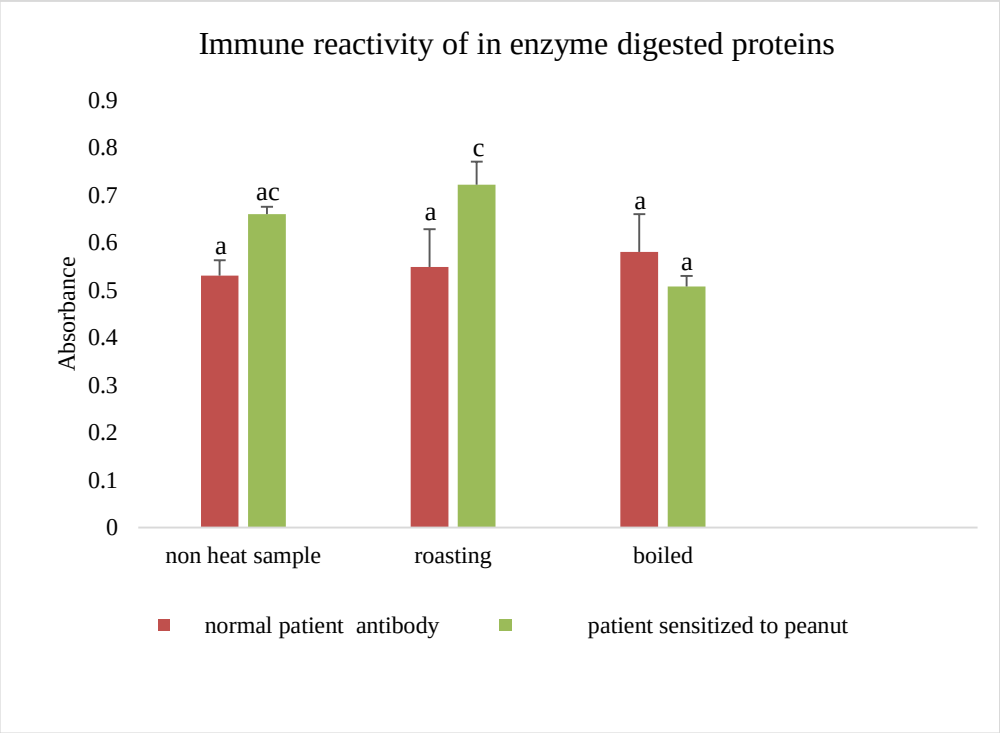

Figure 6.17. Immune reactivity as measured by ELISA of the black seed mix subjected to different thermal treatments followed by enzyme digestion. Heat treatments were non-heated, roasting (standard process), and boiling of peanuts and sesame seeds before addition to the black seed mix. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences ($P<0.05$).

Enzyme digestion did not affect the immune reactivity of proteins extracted from the black seed mix, probably because the epitopes in the protein structure are not accessibly to proteolytic enzymes.

6.4.3 The effect of different concentrations of sodium bisulphite on the immune reactivity

Different concentrations (50, 100 and 200 mg) of sodium bisulphate (SB) was added to 40g of the black seed mix before roasting. Figure 6.18 shows that 50 and 100 mg SB concentrations resulted in increased immune reactivity for both the normal control and the patient sensitised to peanut, whereas 200 mg SB resulted in significantly reduced immune reactivity for both test groups. The explanation could be that reduction disulphide bonds in allergenic proteins such as peanut 11S by SB is concentration dependent, where low concentrations lead to partial unfolding of proteins and exposure of conformational whereas a high concentration would sufficiently disrupt the tertiary structure to destroy the conformational epitopes.

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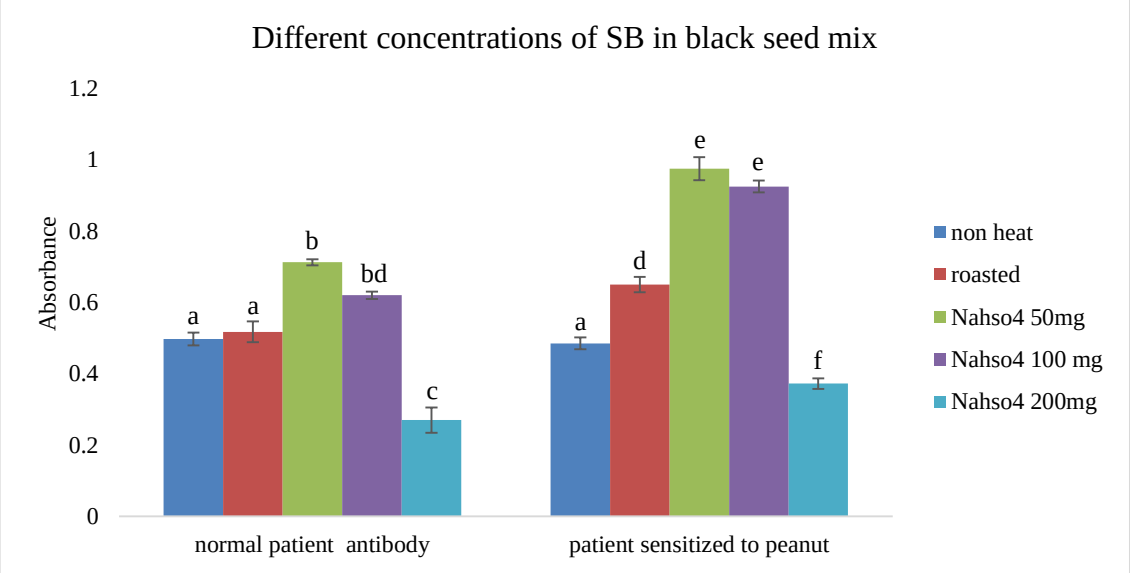


Figure 6.18. Effect of SB concentrations in roasted black seed mix on the immune reactivity as measured by ELISA. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences ($P<0.05$)

6.4.4 Effect of sodium bisulphite and vitamin C in immune reactivity

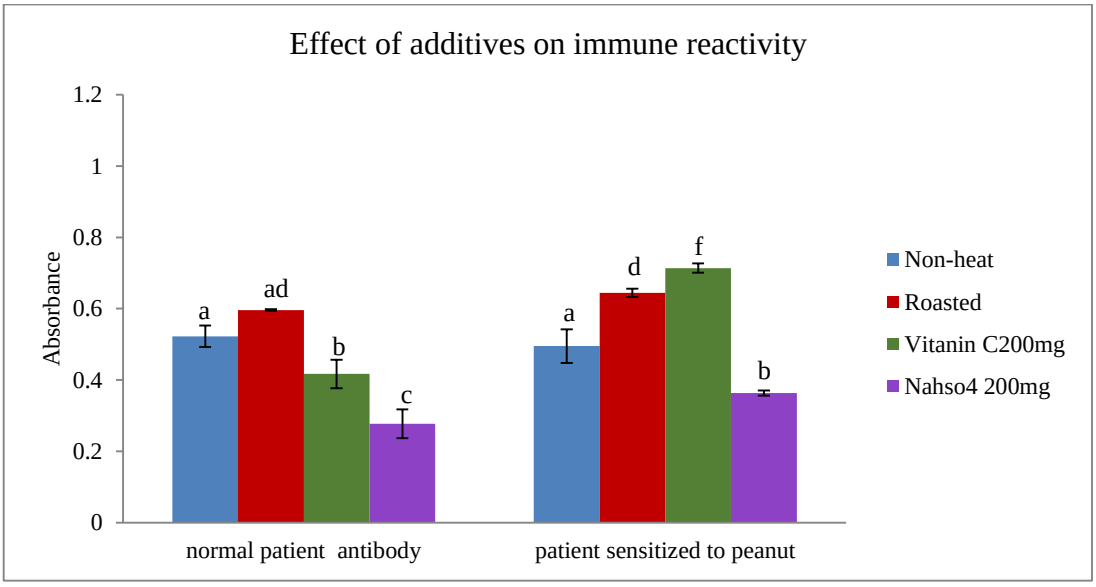


Figure 6.19. Effect of the presence of SB and vitamin C in the sample on immune reactivity using ELISA. Bars are means of three measurements and error bars are standard deviation. Different letters indicate significant differences ($P<0.05$)

Figure 6.19 confirms the results of Figure 6.18 for the reduction of immune reactivity caused by 200 mg SB. On the other hand, the addition of vitamin C significantly increased immune reactivity with respect to the sensitized patient's serum.

We report here for the first time that the addition of the approved food additive sodium bisulphite to a complex matrix such as the black seed mix lowers the immune reactivity of peanut allergens. One of the aims of this study was to test the hypothesis that the addition of a food-grade reducing or oxidation compound could reduce the potency of food allergens. Mattison (2014) pre-empted confirmation of our hypothesis by demonstrating the effect of sulphite on cashew allergens. Whereas Mattison demonstrates the effect in isolated cashew nut allergens, we report the effect on peanut allergens heat-treated in a complex food matrix with sodium bisulphite as an additive. The results confirm the results of Mattison (2014), who reports reduced ELISA results for cashew nut treated with sodium fsulphite. Mattison (2014) interpret their findings as indicating that sulphite

compounds disrupt disulphide bonds and destroy the conformational IgE epitopes that require those bonds, thereby reducing IgE binding. Furthermore, they reason that attachment of the negatively charged sulphite ion to the cysteine residues would repel IgE from linear epitopes and reduce IgE binding. This explanation could be valid for the reduction in immune reactivity by addition of sodium bisulfite to the black seed mix.

To validate the efficiency of sodium bisulphite to reduce nut allergy in humans, further tests such as skin prick tests are necessary. Reductions in *in vitro* IgE binding assays do not always correlate with allergic reactions *in vivo* (Shi *et al*., 2013).

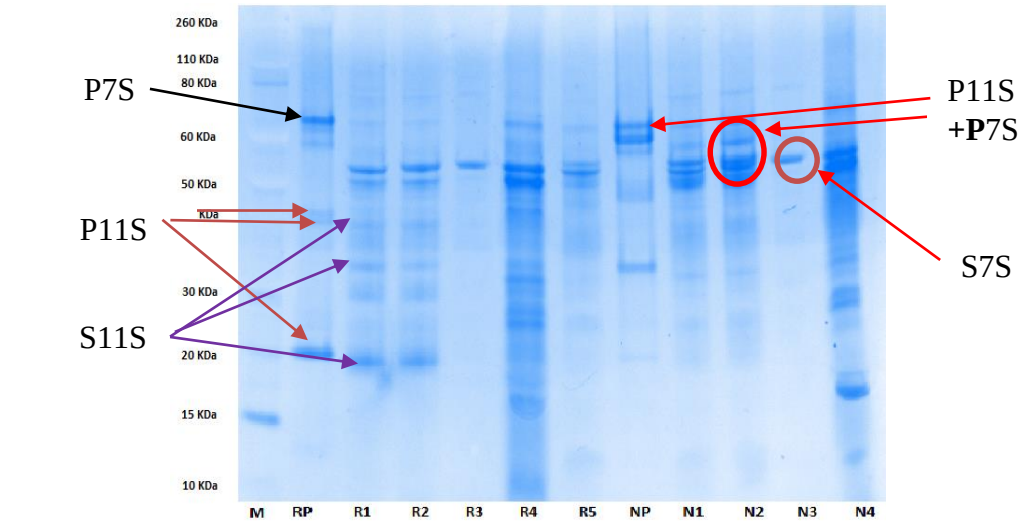
6.4.5 SDS-PAGE and Western blot

6.4.5.1 SDS-PAGE

The results of SDS-PAGE under reducing and non-reducing conditions cover the following aspects:

1. the effect of thermal processing of the black seed mix on protein sub-fractions in the soluble extract and insoluble pellet compared to that of purified peanut protein standard
2. the effect of the addition of sodium bisulphite or vitamin C to the roasted black seed compared to purified peanut protein standards.

A)



B)

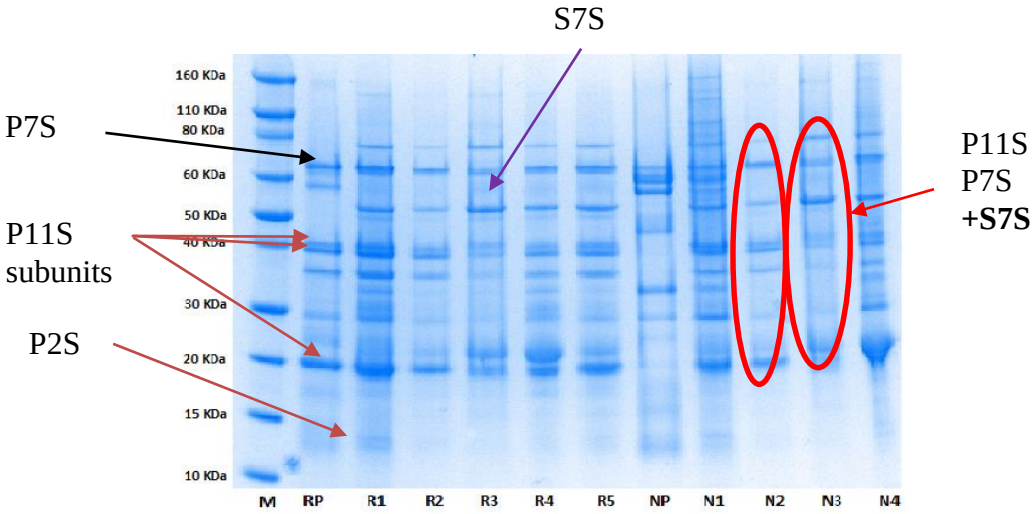


Figure 6.20. SDS-PAGE of the soluble (upper figure **A** and insoluble (lower figure **B** extracts of black seed mix. Lanes 1-7 are reduced samples of: **M** molecular weight markers (RP) peanut protein p, (R1) non heated black seed mix, (R2) roasted black seed mix, (R3) boiled black seed mix, (R4) sodium bisulphate treated, and (R5) vitamin C treated. Lanes 8-12 are non-reduced samples of peanut protein (NP), control (N1), roasted (N2), boiled (N3), and sodium bisulphite treated (N4). The arrows indicate the types of protein: P2S, P7S, and P11S are peanut proteins, and S7S is sesame seed protein

6.4.5.1.1 Effect of roasting and boiling of black seed mix on protein fractions in the soluble extract and insoluble pellet

Figure 6.20 A shows that the soluble extract of the control (N1) and roasted (N2) black seed mix contains both peanut 11S and 7S as well as sesame 7S as marked by the red

circles. However, the boiled soluble extract only contains sesame 7S but not peanut 11S or 7S. This means that boiling of the peanuts and sesame seeds before addition to the black seed mix rendered the peanut 11S and 7S insoluble and not extractable. Bands for peanut 11S and 7S are indicated by arrows in lanes N1 and N2, and the reduced subunits for peanut 11 S (40, 38, and 20 kDa) are visible in lanes R1 and R2, corresponding to the peanut protein standard (NP and RP).

Figure 6.20 B shows electrophoretic patterns of the insoluble pellet of the black seed mix. The proteins were solubilised by treatment with the Ready Prep protein extraction kit (section 2.2.13). Lanes N3 and R3 show the presence of peanut 11S and 7S subunits in the boiled sample confirming that boiled treatment of the black seed mix renders these proteins insoluble and not extractable compared to roasting and no heat treatment. The electrophoretic patterns of the non- reduced pellet (N1-N4) are similar to those of the reduced pellet (R1-R4). The reason is that reducing agents were in the buffer of the Ready Prep protein extraction kit used to solubilise the insoluble proteins. So when the samples were consequently dissolved in SDS-PAGE sample buffer with or without mercapto ethanol for non-reducing and reducing conditions (2.2.13), no differences were observed in electrophoretic patterns.

These results also show that the extraction method used in this study was able to extract some of the 2S, 7S, and 11S peanut globulins for the non- heated and roasted black seed mix preparations the results also confirm that these proteins were present in the samples used for the ELISA assay and determination of Maillard reaction products.

6.4.5.1.2 The effect of the addition of sodium bisulphite or ascorbic acid on roasted black seed mix proteins

The effect of the addition of sodium bisulphite on protein band patterns can be seen in R4 and N4 for both the soluble and insoluble extract (Figure 6.20 A and B). The protein band

patterns for the non-reduced sample (N4) were similar to those of the reduced sample (R4). This provides evidence that sodium bisulphite reduced the disulphide bonds *in situ* in the black seed mix, and confirms the findings for the sulphite reduction of soy 11S (Zhang and Sun, 2008) and cashew nut 11S and 2S (Mattison *et al.* 2014). Whereas these authors demonstrated the effect of sulphite reduction of purified proteins, the results reported here demonstrate the effect in a complex food matrix containing peanuts.

The bands in these lanes were consistently darker than those of the other extracts, despite repeated protein determinations to load the same number of proteins as other extracts on the gel. The reason could be improved solubility of proteins caused by the presence of sodium bisulphite in the black seed mix, which improved the extraction process. Khuda *et al*. (2015) report that the inclusion of reducing agents such as dithiothreitol (DTT) in the extraction buffer resulted in a five-fold increase in protein extraction efficiency.

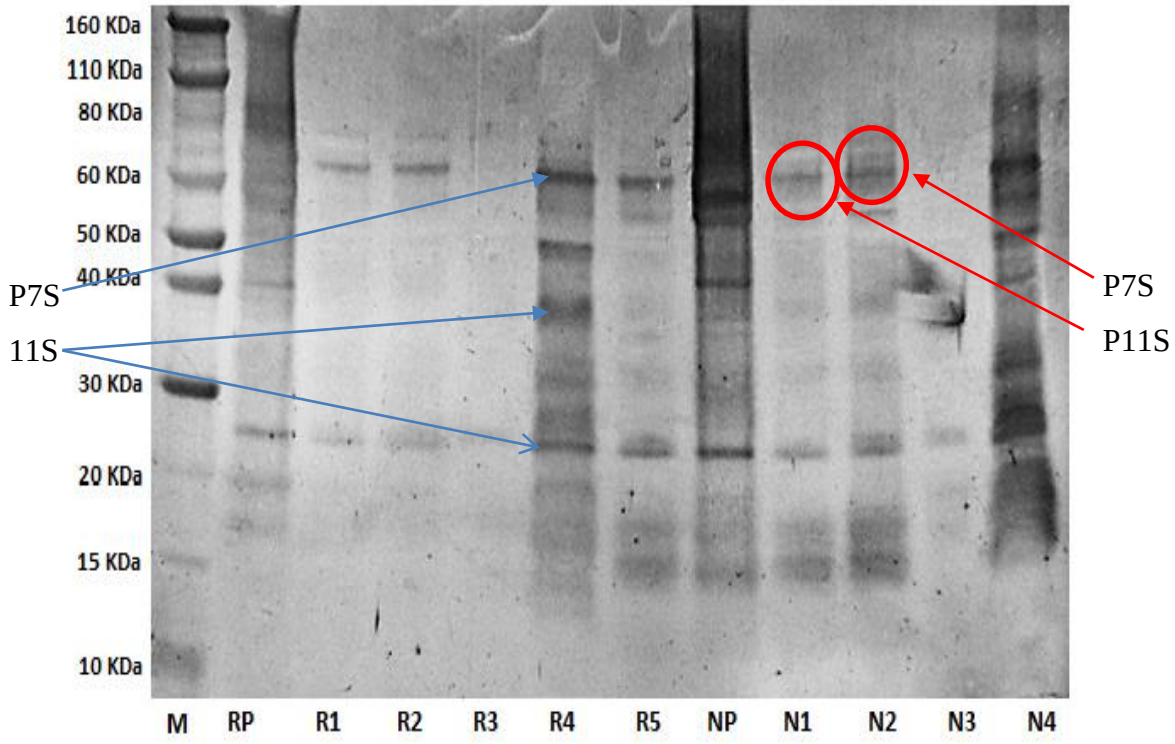
The addition of vitamin C to the black seed mix resulted in less extractable protein, with only sesame 7S and traces of sesame 11S in the supernatant (Figure 6.20 A, lane R5), However, all protein sub-fractions for peanut and sesame were present in the pellet (Figure 6.20 B, lane R5). This indicates that the addition of vitamin C could have accelerated the formation of larger insoluble protein aggregates in the black seed mix, probably by the acceleration of disulphide bond formation by oxidation.

6.4.5.2 Western blot

The results of the Western blot of the respective SDS-PAGE gels above will cover the following aspects:

- The effect of different processing conditions of the black seed mix on the reactivity of the serum IgE of patients allergic to peanuts to protein extracted from the black seed mix.
- The effect of the addition of sodium bisulphite or ascorbic acid to the black seed mix on the reactivity of the serum IgE of patients allergic to peanuts.

A)



BA)

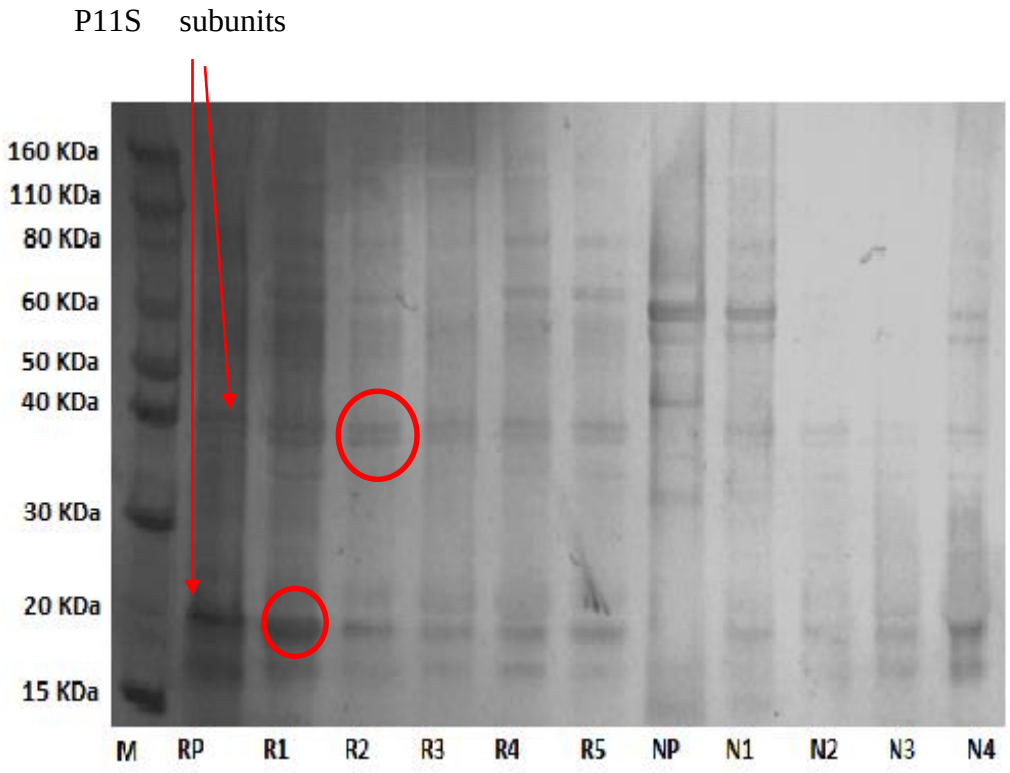


Figure 6.21. Western blot analysis of the supernatant **A** and pellet **B** of the black seed mix protein extract. Lanes 1-7 are molecular weight markers **M**, reduced peanut protein (RP), control (R1), roasted (R2), boiled (R3), sodium bisulphite treated (R4), and vitamin C treated (R5). Lanes 8-12 are non-reduced peanut protein (NP), control (N1), roasted (N2), boiled (N3), and sodium bisulphite treated (N4). The arrows indicate the types of protein: P7S and P11S are peanut proteins

6.4.5.2.1 Effect of processing of black seed mix on the immune reactivity

Figure 6.20 A shows the immune reactivity of peanut P11S and P7S for the soluble extracts of control and roasted samples as indicated by the red circles, whereas these bands were absent for the boiled sample (N3 and R3). These results confirm the reduced reactivity of the ELISA results (Figure 6.16) for the boiled sample. These results confirm the findings of Mondoulet *et al.* (2005) for absence of 7S extracted from boiled peanuts.

The immunoblot of the pellets (Figure 6.21 B) shows visible immune reactive bands for both P P11S in the insoluble pellet of the boiled black seed mix. This confirms that P11S was rendered insoluble by boiling preparation of the black seed mix and was not

extracted by the method used in this study. The bands are heavier for the non -roasted sample (N1 and R1) than for the roasted sample (R2 and N2) and confirm the SDS-PAGE profiles (Figure 6.19 B). The differences in intensity could be due to differences in the concentrations of protein loaded on the gel because of the difficulty of solubilising insoluble proteins from the roasted sample.

6.4.5.2.2 The effect of addition of sodium bisulphite to roasted black seed mix

The sodium bisulphite-treated extract shows increased immune reactivity by Western blot compared to the non-heated sample (Figure 6.21, lane N1 versus N4) and do not correspond to the results of the immune reactivity to 200 mg BS treated black seed mix by the ELISA (Figure 6.19). It also differs from the results of Mattison (2014), who reported decreased immune reactivity of protein extracted form cashew nuts that had been treated with SB. This discrepancy could be due to a higher concentration of protein loaded on the SDS PAGE gel compared to the control (Figure 6.20) caused by improved protein solubility of the extract.

6.5 Discussion

The results presented here provide evidence for significantly reduced immune reactivity for the boiled black seed mix as measured by the ELISA. These results correspond to that of Beyer *et al*., (2001) who reported that boiled peanuts resulted in reduced allergenic response in patients by the skin prick test. Utilizing a peptide array-based immunoassay as well as western blotting techniques the authors demonstrated that the severity of clinical response correlated more to conformational epitopes of P11S and 7S than the linear epitopes. However the author's extracted proteins form peanuts before boiling, whereas in the present study proteins were extracted after boiling and further heat treatment with the ingredients in the black seed mix. Our results of SDS PAGE and Western blot show the loss of peanut 11S and

7S protein in the soluble extract of the boiled black seed mix compared to the non- heated and roasted preparations. The loss of peanut 7S from boiled peanuts was also reported by Mattison *et al.* (2014), who postulated that boiled peanut 7S leaches into boiling water. Our results demonstrate for the first time the loss of both P7S and P11S in the soluble extract of the boiled black seed mix and confirm their presence in the insoluble pellet extract. These results also support the findings of Koppelman *et al.* (1999) and Blanc *et al.* (2011) and who demonstrated that 7S purified from peanuts undergoes extensive aggregation after boiling, resulting in high molecular weight aggregates. The reduced ELISA response of the boiled sample presented here is probably due to the loss of peanut 7S and 11S resulting in a reduced response peanut allergenic patients. The immune reactivity of insoluble 7S and 11S of boiled peanuts still need to be determined by the ELISA. However, this would not be possible as the proteins need to be solubilised by harsh reagents not suitable for the ELISA. It could be assumed though that the extensively aggregated proteins would result in significant loss in conformational epitopes and could result in loss of clinical allergenicity as demonstrated by Beyer *et al*., (2001).

Soluble extract of the roasted black seed mix resulted in significantly increased immune reactivity compared to the non- heated version as measured by the ELISA. This correspond to a more intense colour reaction by Western blot (Figure 6.20 lane N2) although the SDS PAGE indicated similar protein concentration load (Figure 6.19 Lane N2). These results correspond to the evidence of higher concentration of third stage Maillard reaction products in the extracts of the roasted compared to the non- heated black seed mix. We report here for the first time correlation between increased immune reactivity as measured by ELISA and the third stage Maillard reaction products.

Addition of sodium bisulphite to the roasted black seed mix showed a marked reduction in immune reactivity by ELISA, corresponding to increased bisulphite

concentrations (Figure 6.18). However, the western blot showed a significantly more intense reaction with antibodies (Figure 6. 20 Lanes N4 and R4). This could be due to higher sample load on the SDS PAGE gel (Figure 6.19 lanes N4 and R3). Moreover, the immune reactivity of proteins as tested by Western blot alone might not be a true reflection of the status of conformational epitopes, as SDS-PAGE could lead to matrix-induced changes in conformational epitopes (Beyer *et al*., 2001) and might also have resulted in the removal of sulphite ions that mask the epitopes (Mattison *et al.* 2014). Furthermore, previous studies have shown that the level of IgE binding by Western blotting does not necessarily correlate to the severity of clinical allergic response (Hourihane *et al*., 1997). The results confirm the findings for the sulphite reduction of soy 11S (Zhang and Sun, 2008) and cashew nut 11S and 2S (Mattison *et al.* 2014). Whereas these authors demonstrated the effect of sulphite reduction of purified proteins, the results reported here demonstrates the effect in a complex food matrix containing peanuts. Addition of ascorbic acid did not reduce the immune reactivity as measured by the ELISA. We demonstrate here for the first time that a complex food matrix containing peanuts and subjected to boiling resulted in a loss of extractable peanut 7S and 11S, whereas sesame 7S was still extractable. The results demonstrate that different processing methods resulted in the extraction of different protein sub-fractions because of differences in solubility. The results confirm the conclusion of Khuda *et al.* (2005) that the extraction of proteins from a complex thermally processed food matrix for the purpose of physicochemical and immunological analysis still needs to be optimised.

CHAPTER 7

Discussion and Conclusion

7.1 Novelty

The novel findings resulting from this PhD study are as follows:

The development of a sensory-acceptable nutritional product consisting of nuts, seeds, and treacle that is categorised as a chewy, sweet confection and has an acceptable shelf life of at least one month under accelerated storage conditions.

- The product could serve as a source of carbohydrate, fibre, protein, essential amino acids, magnesium, zinc and iron for all age groups
- The non- heated product exhibits excellent radical scavenging, ferric reducing and inhibition of lipid peroxidation properties.

Demonstration of the effects of quantified different stages of MRP as generated by different heating methods of the black seed mix on anti -oxidant properties:

- Roasting resulted in third stage MRP, increased radical scavenging activity and reduced lipid peroxidation inhibition activity
- Boiling of peanuts and sesame seeds before addition to the black seed mix resulted in increased intermediate stage MRPs (reduced third stage MRPs) correlated with increased radical scavenging activity and increased lipid peroxidation inhibition activity
- The increased lipid peroxidation inhibition activity with the boiled black seed mix was similar to that of a synthetic food ant oxidant additive, BHT.

Demonstration of the effect of different thermal processing of the black seed mix on extractable proteins and immune reactivity:

- The boiled mixture yielded a loss of extractable peanut 7S and 11S allergens, which resulted in loss of immune reactivity as determined by ELISA and Western blotting.
- Extracts from the non- roasted and roasted black seed mix contained 7S and 11S allergens.
- Immune reactivity of extracts of the roasted black seed mix was increased and correlated with increased MRPs compared to that of the non- heated product

The addition of the approved food additive sodium bisulphite to the roasted black seed mix lowered the immune reactivity of peanut allergens.

- The application of sodium bisulphite resulted in reduction of the disulphide bonds of peanut proteins as demonstrated by SDS PAGE and concentration dependent reduction in immune reactivity as measured by the ELISA.

7.2 Overview of the results

To summarise the overall outcome of this study: The black seed mix is a chewy sweet confectionary product that provides many essential nutrients. Roasting of the black seed mix resulted in increased third stage MRPs that yielded increased radical scavenging ability, but reduced ability to inhibit lipid peroxidation and increased immune reactivity. Boiling of peanuts before addition to the black seed mix resulted in increased second stage MRPs, increased radical scavenging ability, increased ability to inhibit lipid peroxidation and reduced immune reactivity, due to loss of soluble peanut 7S and 11S. It is concluded that the preferred thermal processing method of the black seed mix would be boiling of the peanuts before addition to the rest of the ingredients and further processing. Addition of sodium bisulfide to the black seed mix before roasting resulted in reduced immune reactivity which was due to reduction of disulfide bonds of the 11 S peanut allergen.

7.2.1 The black seed mix chewy, sweet confection

We report here the development of a chewy, sweet confectionary product as defined by its water activity and sugar content. The texture, as measured by hardness, fell within the range of acceptable texture for a confectionary product in this category, and the sensory properties were acceptable, as shown by sensory evaluation. The results of this study show that 200g of the black seed mix would provide the RDI for all age groups of fibre, linoleic acid,

magnesium, zinc, iron, histidine, isoleucine, methionine, cysteine and valine. Furthermore, it would provide 50% of RDI for protein, fat, carbohydrate, lysine, leucine, phenylalanine and tyrosine for all age groups. Although 200g would be a relatively large portion for small children to consume and a more feasible size would be 20-50g per day, the nutritional value of 200g was used in this study as a uniform quantity for all age groups. Nevertheless, even smaller portions would add significantly to daily nutrition.

An outstanding feature is the high content of iron derived mainly from treacle, which has been demonstrated in previous studies to be biologically available. This identifies the use of black seed mix as an inexpensive nutritional supplement, which would be essential to address the prevalence of anemia in malnourished individuals.

The shelf life of the vacuum-packaged product in accelerated storage conditions (37°C) was acceptable for one month, as determined by the increase in peroxide value of fats after 2 months. This would equate to to 2.5 month's storage at ambient temperature based on published information of a product with similar composition. The hardness and fracturability of the product increased after 1 months' storage, but remained within acceptable limits according to comparisons with data in the literature. The water activity also increased after 1 months' storage but remained within published limits for this category of confectionary. It is theorized that the sugar in the product underwent a phase transition from amorphous to glassy state at a storage temperature of 37°C, which falls within the published range of glass transition temperature for this category of product. The shelf life could be extended by optimising the packaging conditions in the case of large scale manufacture and storage conditions below 30 °C.

Antioxidant tests show that the non- heated product has excellent radical scavenging, ferric reducing and inhibition of lipid peroxidation properties that are probably related to the high phenolic content of the ingredients in the black seed mix. Further

studies showed that the antioxidant properties are affected differently by different thermal processing methods.

7.2.2 Effect of processing on extractable proteins

We report the loss of extractable soluble peanut 7S and 11 S for the boiled black seed mix using SDS PAGE and Western blotting techniques, which resulted in reduced immune reactivity as measured by the ELISA. The loss of peanut 7S was previously reported for boiled peanuts alone but has not been demonstrated in a complex thermally processed food matrix. Comparatively, peanut 7S and 11 S and sesame 7S was readily extractable in the roasted and non- heated black seed mix.

7.2.3 Antioxidant properties of different stages of Maillard reaction products (MRP)

We report the increase in radical scavenging properties for roasted and boiled processing methods that correlated with increased in third stage MRPs. Currently, the radical scavenging activity of antioxidants that are naturally present in food products is regarded as a quality parameter to indicate the dietary value (Ak and Gulcin, 2008). On the other hand, the increased third stage MRPs in of the roasted black seed mix correlated with decrease in inhibition of lipid peroxidation, which corresponds to increase in peroxide value of the roasted black seed mix after 2 month accelerated shelf life study. It is postulated that this phenomenon might be due to decreased phenolic content caused by roasting based on published findings in roasted coffee beans. On the other hand, extract of the boiled black seed mix contained increased levels of intermediate stage MRPs and decrease third stage MRPs that resulted in increased ability to inhibit lipid peroxidation. The ferric reducing activity was not affected by roasting of the black seed mix and slightly but significantly reduced by boiling of the black seed mix, which again could be due

different effects of thermal processing on natural anti -oxidants in the black seed mix. To our knowledge, the effects of quantified different stages of MRP as generated by different heating methods of a plant food system on anti -oxidant properties have not been reported. Although many such studies have been reported on model systems of lysine and reducing sugar complexes, these studies might be relevant to the dairy and egg industry and is less relevant to plant containing foods where phenolic compounds also play a role in antioxidant activity, and might be destroyed by too harsh heating conditions.

7.2.4 Effect of different stages of MRP on the immune reactivity of peanut allergens

Roasting of the black seed mix resulted in increased third-stage MRP and increased immune reactivity as determined by the ELISA. Whereas published reports concern purified peanut fractions that had been heat treated with reducing sugars, our results expand this knowledge in that the results are demonstrated in a complex food matrix, where many other components could affect the results.

However, no correlation can be drawn between the intermediate-stage MRP of extracts from the boiled mix and the reduced immune reactivity shown by ELISA and Western blot, as the concentration of immune reactive proteins were reduced as revealed by SDS-PAGE.

7.2.5 Effect of sodium bisulphite

We postulated that the addition of oxidation or reducing agents to the black seed mix could affect the immune reactivity of proteins because of the prevalence of disulphide bonds in 11S and sulfhydryl groups in 2S allergens. Our results show that the addition of bisulphite to the black seed mix results in breakage of disulphide bonds in the food matrix, as confirmed by SDS-PAGE under non-reducing conditions. This leads to a concentration-

dependent reduction in immune reactivity as measured by ELISA, which confirmed the change in conformational as well as linear epitopes. Mattison (2014) demonstrates the effect of sulphite on isolated cashew allergens. The author reason that the attachment of a negatively charged sulphite ion to the cysteine residues would repel IgE from linear epitopes and reduce IgE binding, our results we confirm the reducing effect on peanut allergens heat treated in a complex food matrix with sodium bisulphite as an additive.

Concerns will continue to be raised to the safety of sodium bisulfite, although it is an FDA and CEN approved food additive, as it has been reported to cause allergy-like symptoms in people with underlying asthma and allergic rhinitis (https://www.allergyuk.org/sulphites-and-airway-symptoms/sulphites-and-airway-symptoms). Nevertheless, the present results provide evidence for the effect of substances that reduce disulphide bonds in allergenic proteins, for reduction *in vitro* immune reactivity. The most common natural reducing agents that should be tested in future research is addition of the amino acid L-cysteine and glutathione released form yeast cells Stauffer (1990). This knowledge could also provide a subject for research in identification of natural reducing agents present in plants and food grade micro -organisms.

7.2.6 Effect of protein extraction methods, Western blot, and ELISA on the assessment of immune reactivity

It was difficult to decide on a suitable method for the extraction of proteins from the complex food matrix for the purpose of testing a range of parameters, such as Maillard reaction products (MRP), antioxidant activity, and peanut immune reactivity. All previous studies on the relation between MRP and peanut immune reactivity involve the extraction of specific peanut allergens from peanuts using high salt and detergents following dialysis and subsequent thermal reaction with sugars and further testing by ELISA. Khuda *et al.* (2005) tested efficiency of various methods for extracting peanut allergens from a

chocolate confectionary product by Western blot only, and concluded that an optimum extraction procedure for the immune detection of peanut allergenic proteins from processed foods has not been established.

Our results show that the extraction method we used was suitable to extract a significant proportion of proteins, including all peanut and sesame protein sub-fractions. However, a significant proportion remained in the insoluble pellet. Our results demonstrated that the ratio of these fractions between pellet and supernatant was influenced by the thermal processing process. The extraction process also resulted in corresponding peanut immune reactivity for both ELISA and Western blot. We conclude that the immune reactivity of insoluble proteins as tested by Western blot alone might not be a true reflection of the effect of processing on conformational epitopes, as SDS-PAGE could lead to matrix-induced changes in epitopes. Moreover, a valid assessment of the immune reactivity of peanut allergens extracted from a complex thermally processed food matrix can only be made by means of ELISA combined with Western blotting of soluble and insoluble proteins.

7.3 Impact

The development of a nutritional, ambient-stable confectionary product with raw materials that are readily available in Middle Eastern and African countries, based on a product consumed traditionally, could contribute significantly to alleviate malnutrition. The current study provides information on the required raw material composition for the sufficient provision of protein, essential amino acids, carbohydrates, magnesium, zinc and iron to children, and a significant nutritional addition for adults. The product is tasty and could assist to alleviate malnutrition in children and adults, especially anaemia that is often associated with malnutrition. Not only does this knowledge provide guidance for local

households to optimise the recipe of the traditional product but it also provides a basis for manufacture of the confectionary product on a large scale to enable distribution for humanitarian or commercial purposes. A literature search shows that the global supply of ingredients is suitable for large-scale manufacture, although fenugreek and the black seed mix could be of limited supply. However, even a 50% reduction of these components in the recipe would still provide adequate nutritional properties. This food product is widely made and consumed in households in Middle Eastern countries, and knowledge and optimisation of its composition could be useful as a source of nutrition.

Knowledge of the effect of the processing of complex food products containing nuts is of value in developed countries, where food allergies are increasing. Moreover, the effect of the addition of sodium bisulphite to reduce peanut allergy could be an important contribution to the alleviation of nut allergy. Further studies are necessary to determine the effects of sulphite on the sensory acceptability of the confection. Furthermore, validation of the efficiency of sodium bisulphite in reducing nut allergy in humans by *in vivo* tests such as skin prick tests is necessary. Addition of sodium bisulfite to reduce allergenicity could be of value in in the short term, and in the longer term could provide a subject for research to find natural reducing compounds in plants and food grade microorganisms.

The results of this thesis contribute to scientific knowledge of the effect of different stages of Maillard reaction products on the radical scavenging, ferric reducing and inhibition of lipid peroxidation properties of a food matrix containing phenolic compounds and other natural anti- oxidants. Indirect evidence suggests that the interaction between natural antioxidants and MRPs plays a critical role in the overall anti- oxidant value of the thermally processed food products and requires further investigation.

A diagram for the preparation of the chewy sweet confection with potentially reduced allergenicity is provided below.

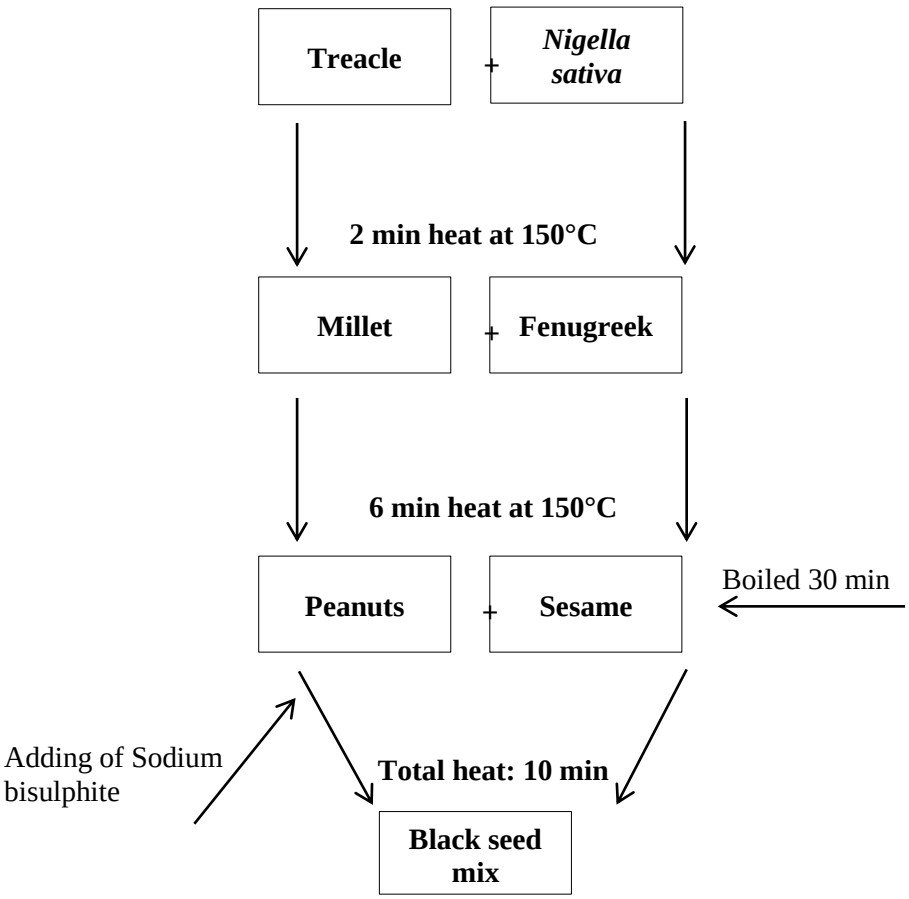


Figure 7.1. Flow diagram for prepared of Black seed mix with low immune reactivity.

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